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13. ABSTRACT *(Maximum 200 Words)*

Both the growth and the development of the mammary gland are controlled by the female hormones estrogen, prolactin and progesterone, and by interactions between the epithelial and stromal compartments of the breast. Changes in the regulation of any of these processes may lead to breast cancer. We have investigated the role of progesterone in the process of sidebranching and alveologenesis in the mammary gland using mice lacking the progesterone receptor which are defective in these processes. By reconstituting murine mammary glands in vivo, we have shown that the progesterone receptor is required only in epithelial cells for proper sidebranching and alveologenesis to occur. Our studies indicate that progesterone acts in a paracrine manner and suggest that Wnt-4 is a mediator of the paracrine signals released from progesterone receptor-positive cells. In addition, we have characterized the role of the estrogen receptor (ER) in regulating the proliferation of breast cancer cells. We postulate that the ability of ER to control cyclin D1 expression and proliferation of breast cancer cells may be acquired during breast cancer progression. This is indicated by the fact that ectopic expression of the estrogen receptor in human epithelial cells does not, on its own, enable signaling between the ER and the cyclin D1 gene. Moreover, others have demonstrated that the ER-positive cells in the mammary epithelium are distinguishable from those that are actively mitotic. Finally, our work indicates that the effects of deletion of the prolactin receptor (PrlR) from mammary epithelial cells closely phenocopy the consequences of deleting cyclin D1 from these cells. We suggest that activation of the PrlR by its ligand results in the production of insulin-like growth factor-2 which in turn induces cyclin D1 synthesis in these cells.

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Final Report
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Our research performed under the auspices of the Army Breast Cancer grant has focused on the ability of three systemic hormones -- prolactin, estrogen, and progesterone -- to govern the proliferation and morphogenesis observed in the mammary gland during puberty and pregnancy. It has been our working hypothesis that deregulation of the cellular pathways normally governed by these three hormones contributes to the pathogenesis of several types of human breast carcinomas.

Prolactin signaling

Our entrée into this problem was created by our finding that breast development was defective in mice that lacked the cyclin D1 gene (1). Such cyclin $D1^{-/-}$ homozygous mutant mice, which we created by homologous recombination, developed reasonably normally in spite of the fact that they lacked the ability to make a cyclin that is usually widely expressed throughout the mammalian body. Among the several, relatively minor morphogenetic defects observed in these mice was a specific and discrete lesion in the morphogenesis of the mammary epithelial tree. In particular, ductal elongation and branching occurred properly during the puberty of female mutant mice. However, during pregnancy, the development of the alveoli was defective.

This defect in alveologenesis represented a deficiency that was specific to the mammary epithelial cells (MECs) within the breast. Thus, when we transplanted cyclin $D1^{-/-}$ MECs into the cleared mammary fat pads of wild type females, the engrafted cells formed ductal trees that were properly branched but failed to form alveoli during subsequent pregnancy of the engrafted female host. This demonstrated that the defect was cell-autonomous rather than representing a systemic defect in the $D1^{-/-}$ female mice.

We imagined that the observed defective alveologenesis reflected the inability of the $D1^{-/-}$ MECs to respond properly to systemic hormones elaborated during pregnancy, notably prolactin. Accordingly, we obtained from others mice that lacked prolactin receptor (PrlR), once again created through a germline knockout of the responsible gene. The phenotype of these mutant mice was complex and difficult to interpret, in part because of some of the mammary gland defects could be attributed to ovarian deficiency and hence to a systemic hormonal defects. However, when we transplanted the $PrlR^{-/-}$ MECs into wild type, cleared mammary fat pads, we observed a phenotype during pregnancy that was quite similar to that seen in the cyclin $D1^{-/-}$ mice (2). From this, we concluded that the PrlR, once activated by Prl in

MECs during pregnancy, induces alveologenesis, and that this alveologenesis depends upon the ability of the MECs to synthesized cyclin D1. Conversely, we concluded that other D-type cyclins (i.e. D2 and D3) are not readily induced by Prl and the PrlR in MECs, explaining the absolute defect in alveologenesis in the breast tissues of the cyclin D1$^{-/-}$ mice.

Following an attractive and simple mechanistic model, the PrlRs displayed on the surface of these MECs, once activated by Prl ligand, could directly induce the expression of cyclin D1 in those cells, which in turn drove their proliferation, enabling them to form alveoli. This model was rendered less likely by our subsequent observation that when Prl ligand was added directly to wild type MECs in culture, cyclin D1 was not induced; other known mitogens, such as EGF and IGF-1, were able to strongly induce cyclin D1 synthesis.

A more complex model proposed that Prl induced the synthesis of one or another type of mitogen in the PrlR-positive MECs, and that this mitogen, once secreted by these MECs, would then act in an autocrine or paracrine fashion on the same or nearby MECs, inducing them to produce cyclin D1. In order to test this model, we resorted to functional genomics, in which we screened the mRNAs expressed by two types of mutant MECs -- PrlR$^{-/-}$ and D1$^{-/-}$ MECs, implanted on contralateral fat pads of the same wild type female mouse which had been induced to enter pregnancy.

These implanted MECs, as expected, exhibited the same defective alveologenesis, and thus served as good controls for one another. However, when we compared the profiles of their expressed mRNAs, we discovered that one mRNA was expressed at ~20-fold lower levels in the PrlR$^{-/-}$ MECs than in the control D1$^{-/-}$ MECs (3). This mRNA, which encodes IGF-2 (insulin-like growth factor-2), provided an explanation for the ability of PrlR to induce proliferation of MECs during the process of alveologenesis.

We are currently examining the possibility that an important mechanism that stimulates the proliferation of MECs during pregnancy can be described as follows: Prl _ PrlR _ IGF-2 _ IGF-2R _ cyclin D1 _ MEC proliferation _ alveologenesis. This model has implications for early steps in breast cancer pathogenesis. Thus, we imagine that it is possible that the ability of MECs within the human mammary gland to acquire the ability to produce either Prl or IGF-2 in a constitutive and autonomous manner leads in turn to a hyperplasia that may enable subsequent progression to carcinomatous growth.

Estrogen signaling

Estrogen plays multiple roles in mammary gland morphogenesis. Ductal elongation is influenced by estrogen (E2) and its receptor (the ER); evidence produced by others indicates that in the case of this specific morphogenetic step, ER-positive cells in the mammary stroma release a mitogen that appears to act upon MECs to elicit ductal elongation. Later in breast development, the ER is expressed in a subset of MECs in the human mammary ductal epithelium, where it is known to induce the synthesis in these cells of the progesterone receptor (PR).

The ER is also known to be expressed in the majority of human mammary carcinomas, where it plays a critical role in mitogenesis. Estrogen applied to the ER-positive cancer cells appears to act as a direct mitogen on these cells, inducing their proliferation. An important question is how E2 and the ER succeed in driving the proliferation of these ER-positive cancer cells. In work completed prior to the initiation of this grant, we demonstrated, as had others, that the ER in human MCF-7 mammary carcinoma cells is tightly coupled to the regulators of cyclin D1 expression. In particular, we demonstrated that reversal of a block to ER function imposed by the E2 antagonist tamoxifen, this being achieved through the introduction of E2 into the growth medium of MCF-7 cells, caused within several hours the upregulation of cyclin D1 gene and protein expression(4). This implied that the ER was in some fashion tightly coupled with the transcription factors that influence cyclin D1 gene transcription.

In work undertaken subsequently under the auspices of the present grant, we sought to determine whether ectopic expression of the ER in other types of human epithelial cells endowed these cells with the ability to respond to E2 by initiating proliferation. In the course of these experiments, we demonstrated that cells of the human HaCaT keratinocyte cell line ectopically expressing ER were able to activate transcription of reporter genes carrying in their promoters EREs (estrogen receptor response elements). Nonetheless, the presence of this ectopically expressed ER did not result in these cells in the activation of cyclin D1 expression (5).

These experiments have led us to conclude that the presence of the ER is necessary but not sufficient for the E2-mediated induction of cyclin D1 and resulting mitogenesis. This raises the question of whether, in the normal mammary gland, E2 is able to act as a direct mitogen of MECs. Indeed, work of others has demonstrated that the subset of MECs in the human breast that are mitotically active are distinct from the subset of MECs that are ER-positive. This has left us, in turn, with the speculation that the ability of E2 to act as a direct mitogen of human MECs represents an acquired ability that is not normally present in the human mammary gland. Stated differently, we believe that early during the progression of ER-positive human breast cancers, the ER in certain ER-positive MECs acquires the ability to signal directly to the mitogenic apparatus through its ability to activate cyclin D1 transcription. The acquisition of this ectopic signaling pathway must involve the expression of certain proteins that couple the ER functionally to the cyclin D1 promoter, or alternatively, the loss proteins that block this coupling in normal MECs. The identity of these proteins remains elusive and is the subject of current, ongoing research. However, once this linkage between the ER and the cyclin D1 promoter is forged, MECs that enjoy this linkage can take advantage of ambient E2 to drive their proliferation, this leading to a localized hyperplasia and, in the longer run, to the development of ER-positive carcinomas in situ.

Progesterone signaling

The availability of genetically altered mice lacking the progesterone receptor (PR) has enabled us to examine the precise role that this receptor and its progesterone (P) ligand play in mammary morphogenesis. These mice, created by others, have severe reproductive failures due to the systemic effects of P, notably those affecting the

ovaries. To deal with these systemic problems, we transplanted the $PR^{-/-}$ MECs into the cleared fat pads of wild type mice, as we had done previously in the experiments described above.

These transplantations allowed us to determine the cell-autonomous defects that were displayed by the $PR^{-/-}$ MECs. In the course of doing these experiments, we observed two distinct defects in mammary morphogenesis. First, while ductal elongation was normal in the mammary gland, the sidebranching by these ducts was severely compromised. Second, alveologenesis was also very defective. We imagine that these two morphogenetic processes, as they occur within the normal breast, may actually be manifestations of a common underlying cellular mechanism. Thus, the formation of sidebranches and the initiation of alveolar bud formation involves processes that are similar topologically, in that they both represent extensions of a main duct in an orthogonal direction (6).

We also undertook control experiments in which we engrafted wild type or a $PR^{-/-}$ mammary fat pad in wild type mice, and then implanted wild type MECs into both. Because both mammary epithelia developed normally, we were able to conclude that the absence of PR expression in the mammary stroma has no apparent effect on ductal morphogenesis. Hence, the PR acts only in the mammary epithelium to influence mammary gland development.

These observations indicated that the presence of the PR was essential to ductal sidebranching but they did not address the issue of whether the PR needed to be expressed within the cells that participate directly in sidebranching or whether, alternatively, PR-expressing cells could enable sidebranching in nearby cells within the epithelium. In order to address this issue, we created chimeric epithelia by implanting mixtures of wild type and $PR^{-/-}$ MECs into wild type fat pads. We were able to distinguish the resulting ductal trees in subsequent wholemount analyses because one or the other type of MEC carried a beta-galactosidase marker gene. The behavior of these chimeric epithelia indicated clearly that if a PR-positive MECs were located near to $PR^{-/-}$ MECs, then the latter could participate in ductal sidebranching. This proved that the PR-positive cells release some factor or factors that acts in a paracrine fashion on nearby cells to enable them to undertake sidebranching (6).

Subsequently, we were able to develop evidence that makes it highly likely that this progesterone-induced paracrine factor is a member of the Wnt family of morphogens, and very plausibly Wnt 4. In one set of experiments, we examined the consequences of ectopically expressing the Wnt-1 protein (which acts on cells similarly if not identically to Wnt 4) in a $PR^{-/-}$ mammary epithelium. This ectopic expression was achieved through the use of transgenic mice in which expression of the Wnt-1 reading frame is driven in MECs by an MMTV (mouse mammary tumor virus) transcriptional promoter. The ectopically expressed Wnt-1 protein was able to elicit ductal sidebranching in epithelia arising from $PR^{-/-}$ MECs, largely reversing the

sidebranching deficiency that these $PR^{-/-}$ MECs previously exhibited (7). We concluded from this that a Wnt protein acting similarly to Wnt-1 functions downstream of P and the PR to elicit sidebranching. Indeed, we could induce ectopic sidebranching in wildtype MECs that co-resided within a chimeric epithelium with MECs deriving from the Wnt-1/MMTV transgenic mouse. This confirmed that this Wnt, and by implication other similarly acting Wnt proteins, could elicit sidebranching by acting in a paracrine manner on nearby MECs, thereby behaving similarly to the unidentified paracrine factor that was able, in earlier experiments, to evoke sidebranching from $PR^{-/-}$ MECs.

In fact, our further work provided substantial evidence that the Wnt protein responsible for sidebranching during the early phase of mammary gland morphogenesis is Wnt4. The peak of expression of this protein in the mammary gland coincides roughly with the period during which the bulk of ductal sidebranching is occurring. Moreover, our studies of $Wnt4^{-/-}$ mice prepared by others supported this identification. Initially, the study of the effects of the $Wnt4^{-/-}$ genotype was complicated by the fact that such homozygous mutant mice die late in embryogenesis. Responding to this, we rescued the mammary anlagen from wild type and $Wnt4^{-/-}$ embryonic day 14.5 mice and implanted these anlagen in cleared mammary fat pads. The resulting engrafted epithelia developed as expected if the donor cells were of wild type origin; if, on the other hand, $Wnt4^{-/-}$ MECs were implanted, then sidebranching was severely impaired during the early phase of pregnancy. Later in pregnancy, sidebranching began to occur, ostensibly being triggered by one of the other Wnts that begins to be expressed at this time (7) .

Taken together, these observations indicated that a Wnt protein is both necessary and sufficient for ductal sidebranching during a specific time window in pregnancy. Our evidence suggests that Wnt4 is the major Wnt protein responsible for this, a conclusion that has been strengthened by our observations using *in situ* hybridization that there is a reasonable congruence between the expression patterns of PR and of Wnt4 in the developing mammary ductal tree.

The simplest mechanistic model emerging from these studies is that the functional activation of the PR by P results in the elaboration by the PR-positive cell of a paracrine factor, specifically Wnt4. This factor, once released, then proceeds to trigger sidebranching in nearby MECs, independent of whether the latter also express the PR. Further support of this model has also derived from our observation that exposure of PR-positive MECs in culture to P resulted in a 2-3-fold induction of Wnt 4 mRNA, but we observed no effects on the expression by these cells of the related Wnt-5a, Wnt-5b and Wnt-6 RNAs (7).

In contrast to the actions of prolactin and estrogen, described above, the actions of progesterone do not suggest any specific signaling mechanism that may yield a deregulation of proliferation of the sort that is associated with mammary carcinomas. Hence, it may be the case that this progesterone signaling pathway is of importance

to mammary morphogenesis but not carcinogenesis. Having completed this work, we have ceased further exploration of this signaling pathway.

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Prolactin Controls Mammary Gland Development via Direct and Indirect Mechanisms

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The inactivation of the prolactin receptor gene by homologous recombination has made it possible to investigate the role of prolactin signaling in mammary gland development without resort to ablative surgery of the endocrine glands. In knockout mice lacking the prolactin receptor, mammary development is normal up to puberty. Subsequently, the ducts branch less frequently than those of wild-type animals. While terminal end buds differentiate to alveolar buds in wild-type females by the end of puberty, in knockout females terminal end bud-like structures persist at the ductal ends. To distinguish between the developmental defects that are intrinsic to the epithelium and those that result from systemic endocrine alterations in prolactin receptor knockout mice, mammary epithelium from prolactin receptor knockouts was transplanted into mammary fat pads of wild-type mice. In virgin mice, the knockout epithelial transplants developed normally at puberty, indicating an indirect effect of prolactin on ductal development. Prolactin receptor knockout females are infertile due to multiple reproductive defects, but epithelial transplants allowed us to assess the extent to which the absence of prolactin receptor is limiting, under systemic conditions that allow full mammary gland development. During pregnancy, the prolactin receptor knockout transplants showed normal side branching and the formation of alveolar buds, but no lobuloalveolar development. Thus, prolactin affects mammary morphogenesis in two different ways: it controls ductal side branching and terminal end bud regression in virgin animals via indirect mechanisms, but acts directly on the mammary epithelium to produce lobuloalveolar development during pregnancy. © 1999 Academic Press

Key Words: prolactin; prolactin receptor; development; mammary gland; tissue recombination.

INTRODUCTION

The mouse mammary gland develops in discrete stages. *In utero*, a rudimentary ductal structure is produced. During puberty the resulting ducts elongate and bifurcate to fill the mammary fat pad, and ductal side branching and the formation of alveolar buds occur during each estrous cycle (Vonderhaar, 1988). During pregnancy, the alveolar buds give rise to lobuloalveolar structures capable of milk production. Following pregnancy and estrus, the gland undergoes involution with loss of most, but not all, of the epithelial components gained during the preceding event. The gland involutes further with declining ovarian function in later life.

A number of hormonal factors control these morphogenetic steps. Embryonic mammary epithelium develops independent of ovarian and pituitary influence (Raynaud, 1971) but is already responsive to hormonal stimuli (Ceriani, 1970). Hormonal replacement in hypophysectomized, ovariectomized, and adrenalectomized mice showed that the development of the mammary ducts is produced by a combination of growth hormone and estrogen. The addition of progesterone to this regimen causes side branching, while alveolar development resembling that of pregnancy requires the addition of prolactin (Nandi, 1958). These hormone combinations were shown to produce similar results using serum-free *in vitro* culture of whole mammary glands, although mammary development did not achieve the extent seen *in vivo* (Ichinose and Nandi, 1964; Vonderhaar, 1988), suggesting that indirect systemic effects of these hormones are important for full development.

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Targeted inactivation of genes in the mouse germ line allows dissection of the respective contributions of various hormonal factors to mammary morphogenesis. (Hennighausen and Robinson, 1998). Moreover, the application of tissue recombination techniques makes it possible to dissect the systemic effects of gene inactivation from direct effects on the target mammary tissue. In this way, it has been shown that the estrogen receptor expressed in the mammary stroma is essential for ductal elongation while its presence in the epithelium is not required at this stage (Cunha, 1997). Conversely, the progesterone receptor is required in the epithelium but not the stroma in order for ductal side branching and alveolar development to occur (Brisken, 1998).

Prolactin and other lactogenic hormones such as placental lactogen may affect mammary development directly via interaction with the prolactin receptor (PRLR), a transmembrane protein belonging to the cytokine receptor superfamily (Bazan, 1989), which is displayed by mammary epithelial cells. Prolactin may also act indirectly via its ability to regulate the function of other endocrine organs responsible for producing mammotrophic factors. Once released, these factors may act in synergy with prolactin to control mammary development. In the ovaries, for instance, prolactin and related lactogenic hormones provide trophic support to the corpora lutea, allowing estrogen and progesterone production (Galosy and Talamantes, 1995). In the liver prolactin regulates the output of insulin-like growth factor-1 (Wennbo, 1997).

Genetic ablation of the PRLR results in mice which show multiple defects in reproduction leading to infertility, altered maternal behavior, and reduced bone development (Ormandy, 1997a; Lucas, 1998; Clement-Lacroix, 1999). In the present work, we have utilized these mice to examine the role of prolactin-mediated signaling in mammary gland development. Tissue transplantation techniques were exploited to determine which of the observed abnormalities in the knockout mice can be ascribed to a direct effect of prolactin on the mammary epithelium and which are due to the loss of the PRLR from other tissues of the mouse.

MATERIALS AND METHODS

PRLR-Deficient Mouse

The PRLR-deficient mice were generated by replacement of exon 5 (Ormandy, 1998), which encodes cysteine residues essential for ligand binding and receptor activation with the NEO cassette (Ormandy, 1997a). Knockout (PRLR$^{-/-}$), heterozygous (PRLR$^{+/-}$), and wild-type (PRLR$^{+/+}$) mice used in these experiments were derived from chimeric animals made using E14 embryonic stem cells (129/OlaHsd) bred to either 129/SvPas or C57Bl6 mice and were housed in 12-h day/night cycle at 22°C and 80% relative humidity with food and water *ad libitum*.

Histology

Tissues were fixed in 10% neutral buffered formalin. Whole mounts were performed as described (Medina, 1973) using hematoxylin or carmine alum staining. Formalin-fixed specimens and whole-mount specimens soaked in toluene to remove methylsalycilate were paraffin embedded and serially sectioned at 5 μm prior to hematoxylin–eosin (H&E) staining. Specimens were photographed and analyzed using a Leica MZ-12 or Leica DMRB microscope fitted with a Sony 3CCD video camera coupled to a Leica Q500MC image analysis program running on a PC.

Transplants of Mammary Epithelium

Transplants were performed as described (DeOme, 1959). Briefly, mammary gland fragments of .1 mm diameter from 8-week-old 129Ola/12SVPas knockout or wild-type mice were transplanted into the cleared fat pads of 3-week-old 129SV/C57Bl6 RAG1$^{-/-}$ mice (Mombaerts, 1992) purchased from The Jackson Laboratory (Bar Harbor, ME). Transplants were analyzed by whole-mount microscopy and histology at 10 weeks after surgery or within the first day postpartum.

Terminology

Mammary gland structures are described using the terminology developed for the human breast by Russo and Russo (1987).

RESULTS

Mammary Gland Development in Knockout Animals

At birth, wild-type and knockout females show rudiments of indistinguishable mammary ductal architecture (data not shown). These rudiments grow slowly until the onset of puberty when terminal end buds (TEBs) form and ductal elongation and bifurcation begin. Examination of the mammary glands at 14 weeks of age (Figs. 1A and 1B) shows reduced ductal side branching in knockout females. In wild-type virgins, the degree of ductal side branching increases with age (compare Figs. 1A and 1C), but in knockout animals, the ductal complexity achieved by 14 weeks remained unchanged at 32 weeks (compare Figs. 1B and 1D). Moreover, by 14 weeks of age, the TEBs of the major mammary ducts and side branches in wt animals had differentiated to yield alveolar buds (Fig. 1A). In the knockout females, TEB-like structures persist at the tips of most ducts (Fig. 1B), some of them being present at the ends of minor ducts as late as 32 weeks of age (Fig. 1D).

The persistent TEB-like structures seen at 14 weeks of age (Figs. 2B and 2D) show no resemblance to the alveolar buds seen at the ductal termini of wild-type animals at this time (Figs. 2A and 2C). Like the typical TEBs seen during ductal elongation they show direct contact between apical epithelial cells of the TEBs and stromal fat cells; however, the TEB-like structures were much smaller and contained far fewer apical cell layers with no distinct cap cell layer. These histological observations reflect their dormant behavior and indicate that the persistent TEB-like structures of knockout females were atypical.

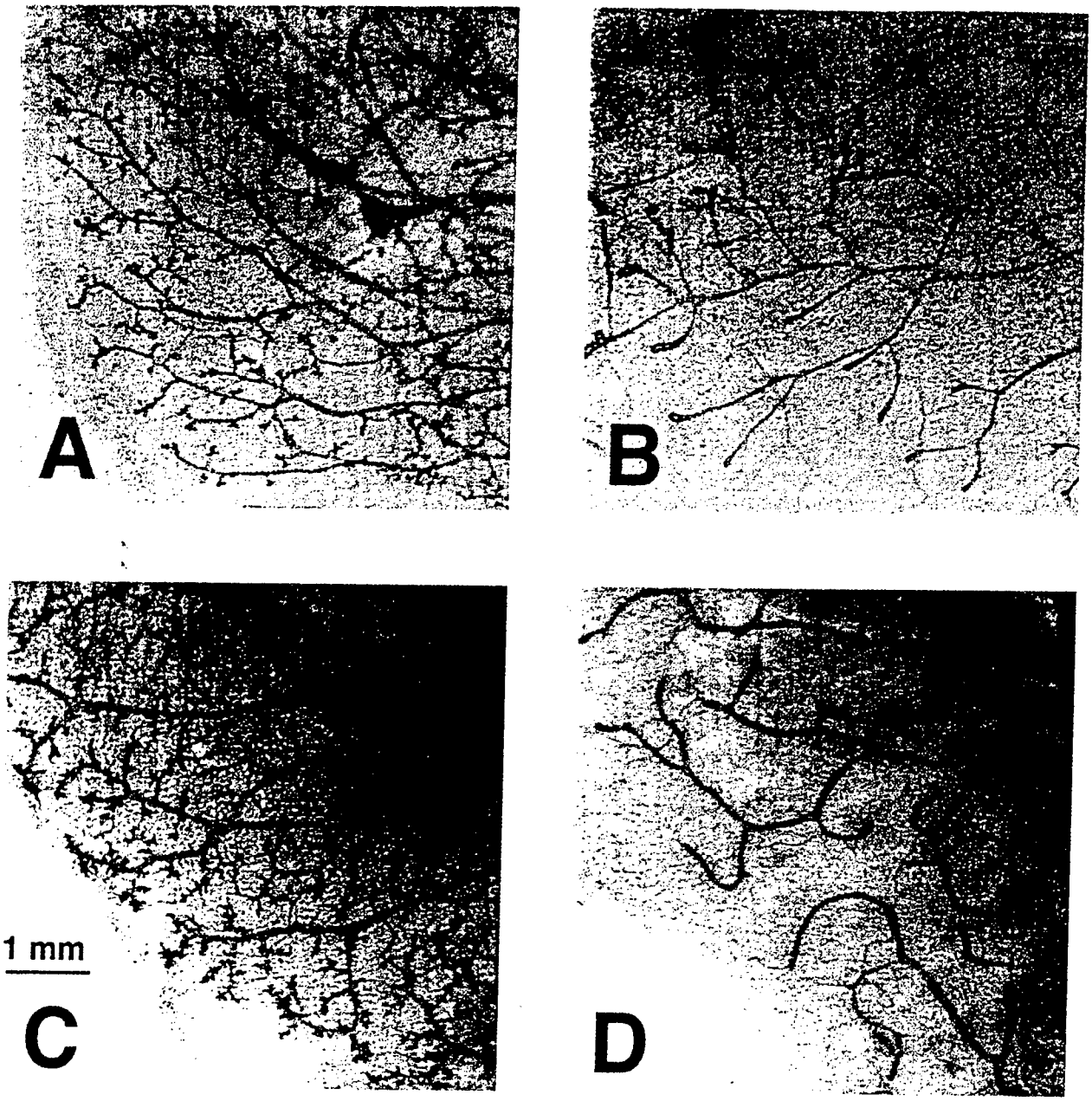

FIG. 1. Whole-mount analysis of mammary development in wild-type and knockout mice. Whole mounts of mammary glands from mice at 14 (A and B) and 32 weeks (C and D) of age were prepared from wild-type (A and C) or knockout (B and D) animals as described under Materials and Methods. Images show the dorsal portion of the fourth inguinal mammary gland. Bar indicates original size.

The male mammary gland was also investigated in animals from these litters (data not shown). No differences were observed between wild-type and knockout males in the proportion of animals with a rudimentary mammary ductal system (7/12 wild-type vs 8/13 knockout with epithelium) or the extent of ductal development.

Mammary Development in Heterozygous (PRLR$^{+/-}$) Animals during Pregnancy

Heterozygous (PRLR$^{+/-}$) females, carrying just one targeted allele of the PRLR, undergo a normal pregnancy but are unable to lactate following their first pregnancy. This effect is generally lost following the second pregnancy

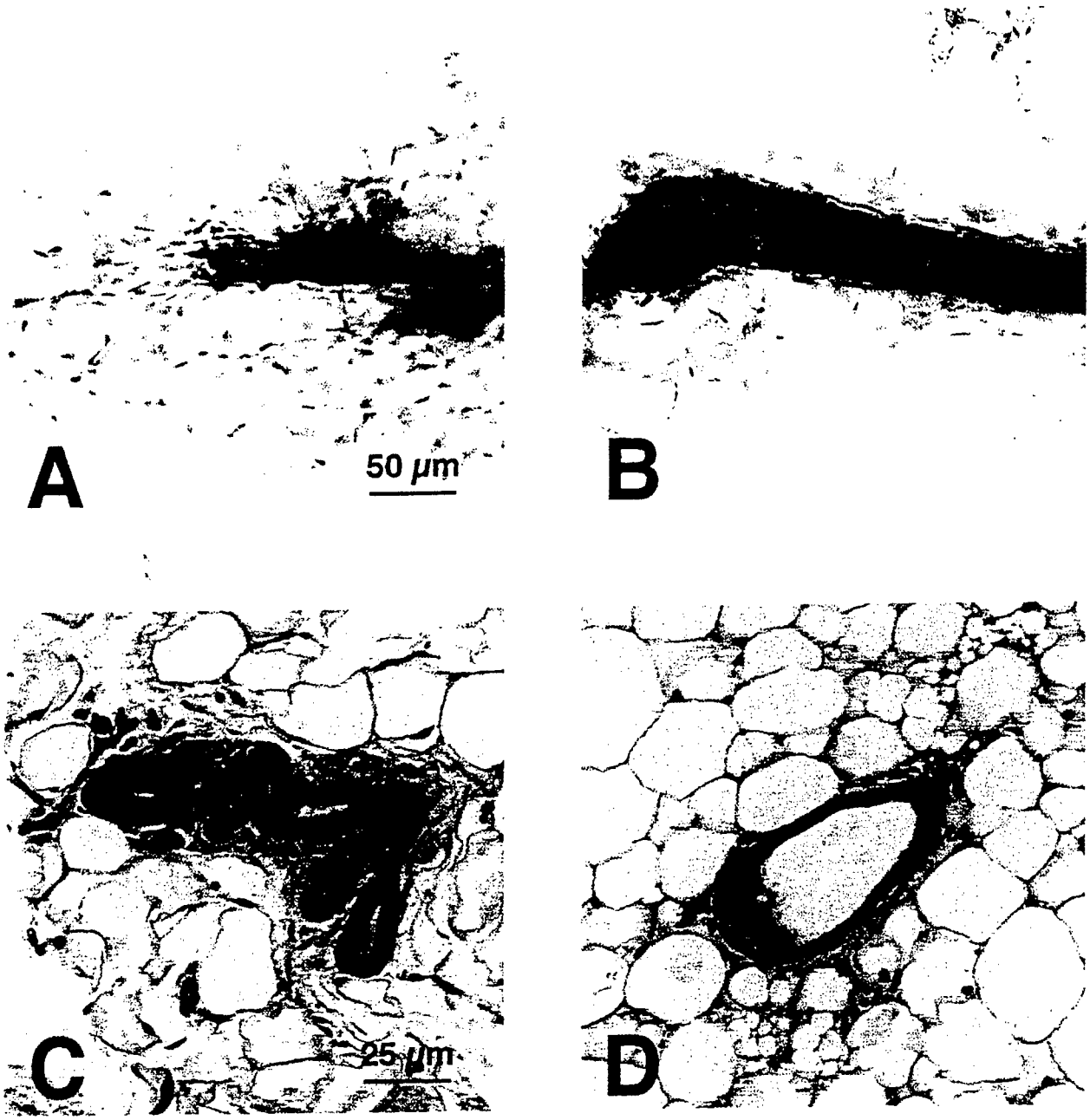

FIG. 2. Terminal ductal structures in wild-type and knockout mice. Ductal termini from whole mounts of mammary glands of virgin 14-week-old wild-type or knockout animals were microdissected and photographed at 200× original magnification under a conventional microscope in a drop of methysalycilate. (A) Ductal termini from wild-type animal showing typical alveolar buds. (B) Ductal terminus from a knockout showing persistent TEB-like structure. Terminal ductal structures from were also examined using H&E staining of 5-μm sections form wild-type (C) showing a typical alveolar bud or knockout (D) showing a TEB-like structure. Bars indicate magnification.

(Ormandy, 1997a). The basis for this observation was investigated using whole mounts and H&E-stained histological sections. Up to midpregnancy, ductal elongation, branching, and the number of lobules formed were indistinguishable between heterozygous and wild-type genotypes (data not shown). However, by day 15 of pregnancy, a substantially greater development of the lobuloalveoli became apparent in the wild-type females (Figs. 3A and 3B). By 1 day postpartum, the mammary glands of heterozygous females showed mostly lobules of stage 2 and 3, with a few lobules of stage 4 at the periphery of the fat pad. In contrast, the mammary glands of wild-type females contained fat pads

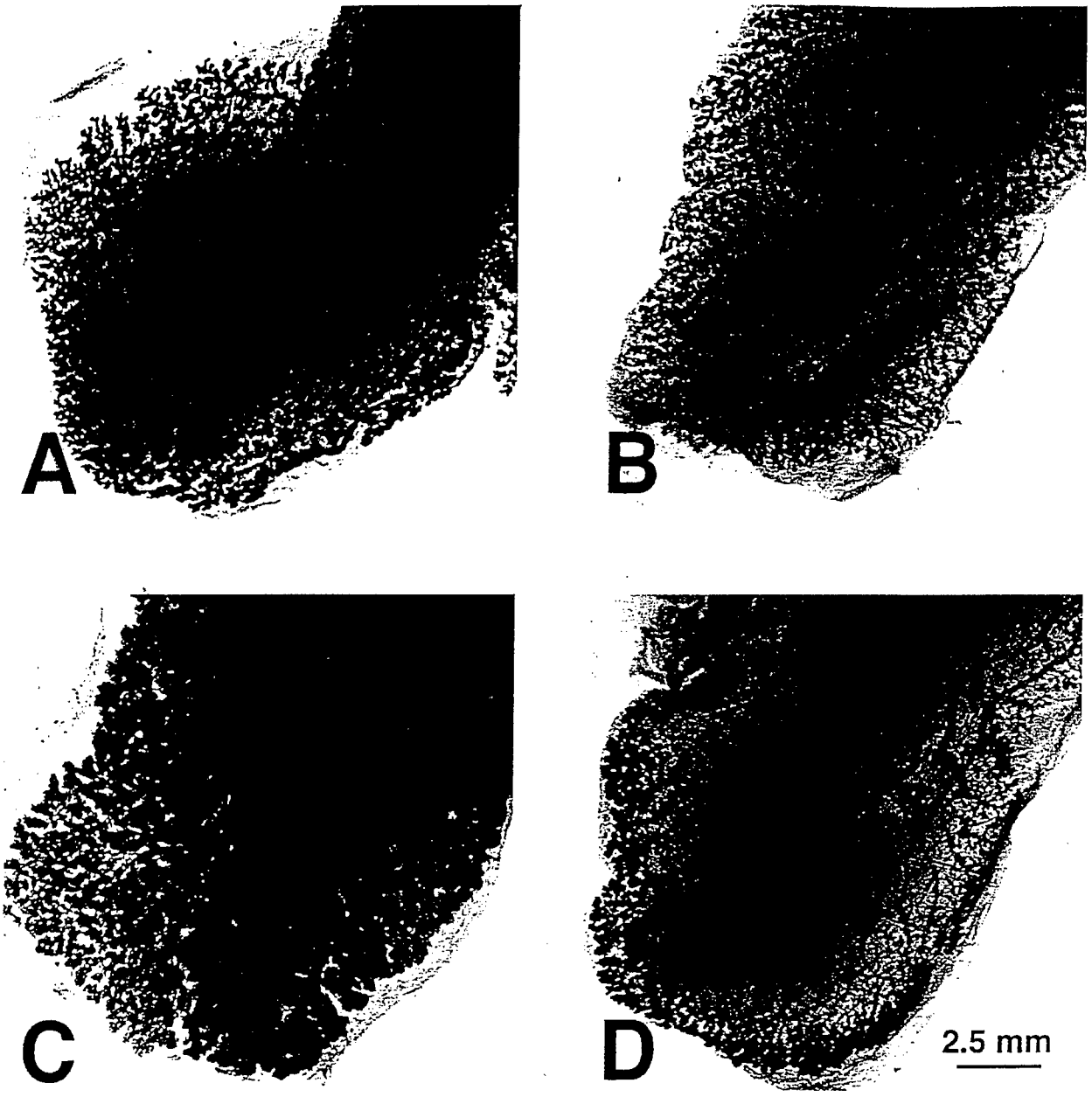

FIG. 3. Mammary development in PRLR heterozygous and wild-type animals during their first pregnancy. Wild-type animals (A and C) or heterozygous ($PRLR^{+/-}$) animals (B and D) were mated at 6 weeks of age and their mammary glands were analyzed by whole-mount histology at day 15 of pregnancy (A and B) or 1 day postpartum (C and D). Observation of the mother's nipples and stomach contents of the pups showed that the heterozygous animals failed to lactate despite the pups attaching to the nipple, while the wild-type animals were able to lactate fully. Bar indicates magnification.

that were densely packed with stage 4 lobules (Figs. 3C and 3D). This phenotype is not fully penetrant, with some females capable of partial lactation following their first pregnancy. The mammary glands of these heterozygous females showed many more stage 4 lobules than the glands of animals unable to lactate, but fewer than seen in wild-type females (data not shown).

Microdissection of stage 3 lobuloalveoli from the periphery of mammary glands from nonlactating heterozygous females 1 day postpartum revealed the formation of multiple alveoli, but unlike wild-type alveoli (Fig. 4A), these alveoli failed to engorge with milk (Fig. 4B). Analysis of H&E-stained serial sections (Figs. 4C and 4D) demonstrated that although the diameter of the alveoli in

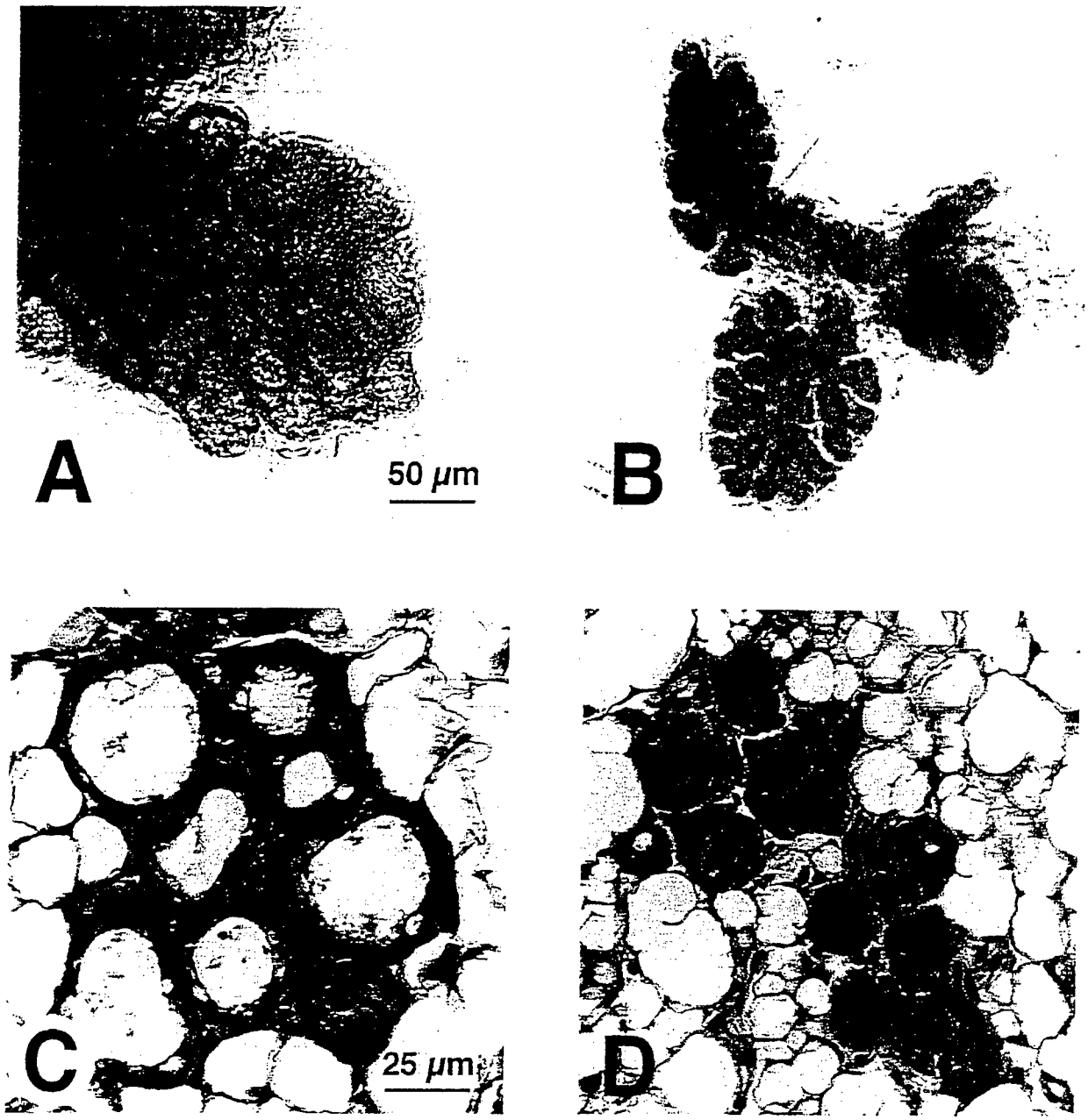

FIG. 4. Histology of mammary alveolar lobules from PRLR heterozygous and wild-type animals 1 day postpartum. Lobules from whole mounts of wild-type or heterozygous (PRLR$^{+/-}$) mammary glands were microdissected and photographed as before. (A) Wild-type lobuloalveoli type 4, 1 day postpartum, lactating normally. (B) Heterozygous lobuloalveoli type 3, unable to lactate, 1 day postpartum. The lobular histology was also analyzed using H&E staining of 5-μm sections. (C) Wild-type lobuloalveoli type 4, lactating normally. (D) Heterozygous lobuloalveoli type 3, unable to lactate. Bars indicate magnification.

heterozygous animals was smaller, they contained a similar number of epithelial cells to those of wild-type animals, indicating a failure of the final stage of functional differentiation, supported by the observation that the heterozygous alveoli contained no secretions and the epithelial cells lining these alveoli gave no evidence of the intracellular vacuoles associated with secretory activity (Figs. 4C and 4D). These results indicate that the loss of one copy of the prolactin receptor gene causes retarded mammary development, rather than a block at a

FIG. 5. Transplantation of wild-type and knockout mammary epithelium to virgin host animals. Whole-mount preparations of mammary glands from virgin RAG1 recipients 10 weeks after surgery. (A) Transplanted knockout mammary epithelium. (B) Transplanted wild-type mammary epithelium. (C) Endogenous fifth inguinal mammary gland. Bar indicates magnification.

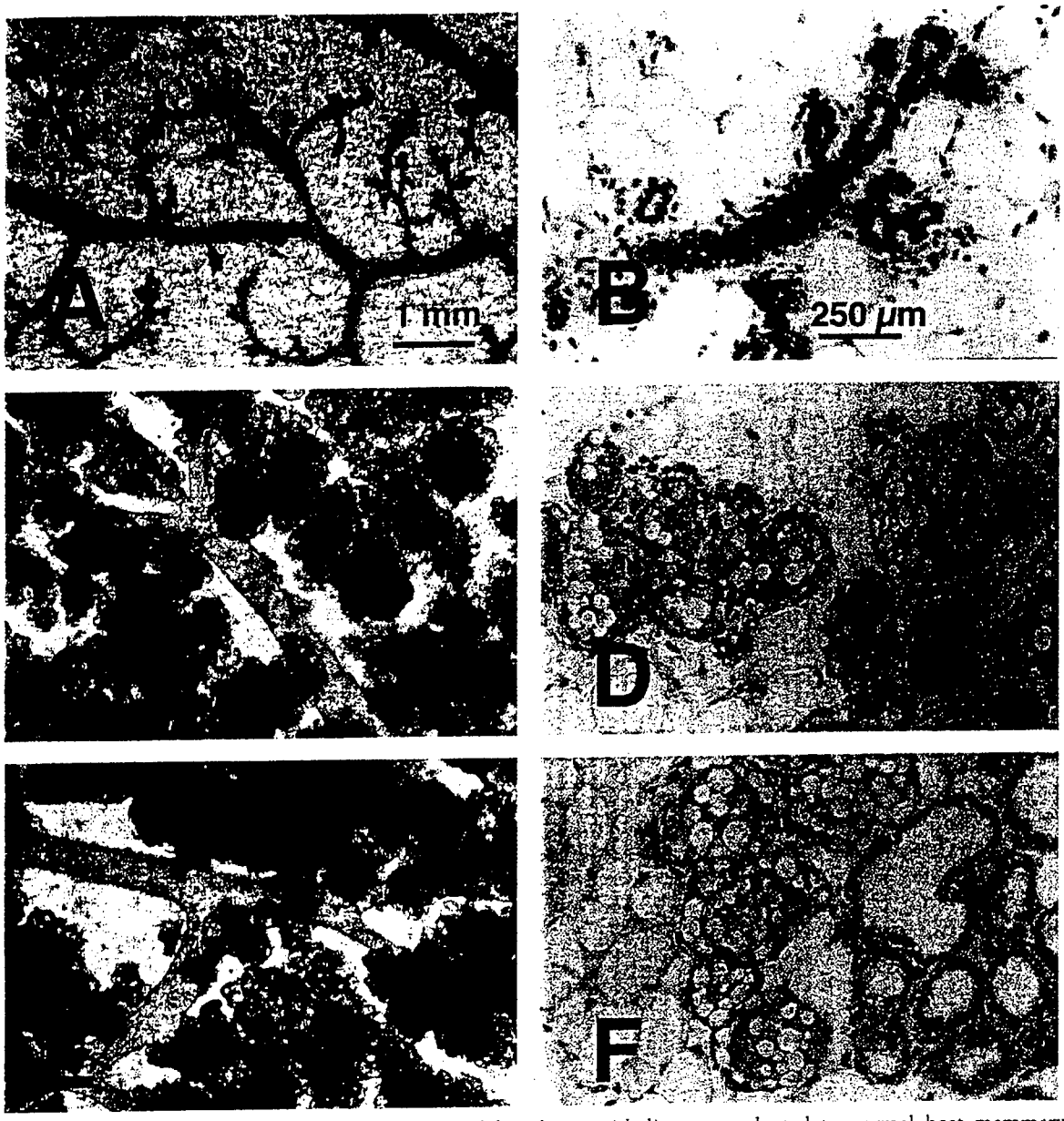

FIG. 6. Whole-mount analysis postpartum of wild-type and knockout epithelium transplanted to normal host mammary fat pad. Mammary epithelium from an 8-week-old knockout animal (A and B) or wild-type animal (C and D) was transplanted into the fourth inguinal mammary fat pad or contralateral fat pad of a 3-week-old RAG1 $^{-/-}$ recipient. The recipient was mated at 10 weeks, and transplants, and an endogenous fifth inguinal mammary gland (E and F), were analyzed using whole-mount histology (A, C, and E) and sections stained for casein (Briskin, 1998) (luminal red-brown staining) (B, D, and F) on the first day postpartum. Bars indicate magnification.

particular stage of development, in contrast to the situation in knockout animals (see below). Given that most of the mammary epithelial component involutes following weaning, the effect of a first failed lactation to enable a second successful lactation is presumably exerted by a cell population which survives involution. The proposed lobular stem cell represents a good candidate population (Kordon and Smith, 1998).

Contribution of the PRLR Outside of the Mammary Epithelium to Pubertal Development of the Mammary Gland

Knockout females show multiple reproductive abnormalities (Ormandy, 1997a) indicative of a wide range of endocrine disturbances. To determine whether the abnormalities in ductal development observed in the knockout females could be ascribed to the lack of the PRLR in the

mammary epithelium or were secondary to defects in other endocrine organs, we transplanted wild-type and knockout mammary epithelia into RAG1$^{-/-}$ recipient females. Mice homozygous for the inactivated RAG1 allele lack T and B cells and are therefore able to accept allografts (Mombaerts, 1992). Both inguinal mammary glands of the recipients were cleared of endogenous epithelium (DeOme, 1959). One side was engrafted with knockout epithelium while the contralateral fat pad was implanted with wild-type epithelium from a littermate. Ten weeks after surgery, the transplanted mammary glands as well as an endogenous gland (to control for a normal endocrine milieu) were analyzed by whole-mount microscopy (Fig. 5).

Whole-mount analysis of a series of seven successfully engrafted mice showed no differences in ductal branching between the engrafted knockout and the wild-type epithelium (compare Figs. 5A and 5B) as well as the endogenous epithelium (Fig. 5C). The TEBs had regressed in all the glands and the ductal complexity was comparable. This indicates that the PRLR is not required in the epithelial cells of the mammary gland in order for ductal development and TEB regression to occur normally. Hence, the effects on these processes observed in knockout females (Figs. 1 and 2) can be ascribed to the absence of the PRLR in other cell types or organs of the mouse.

Development of Knockout Mammary Glands during Pregnancy

The function of the PRLR expressed in the mammary epithelium in mammary gland development during pregnancy could not be assessed in knockout females as these animals are infertile (Ormandy, 1997a). To circumvent this problem, we resorted once more to transplanting knockout and wild-type epithelia into RAG1$^{-/-}$ recipients. The engrafted animals were mated 4 weeks after surgery. The recipients were sacrificed after they had given birth. Both of the transplanted glands and an endogenous gland were analyzed by whole-mount and H&E histology.

In a series of 12 successfully engrafted animals, the wild-type implants (Fig. 6C) displayed a degree of ductal branching and alveolar proliferation comparable to that seen in the unmanipulated endogenous glands (Fig. 6E). The knockout transplants showed extensive side branching but no lobuloalveolar development occurred (Fig. 6A). Histological sections of transplanted and endogenous glands show that the transplanted wild-type epithelium, like the endogenous epithelium, gave rise to functional alveolar structures as indicated by the presence of secretory material in the alveolar and ductal lumina and secretory vacuoles in the epithelial cytoplasm (Fig. 6D). These morphological features of functional differentiation were completely absent from the knockout epithelium (Fig. 6B), and this was confirmed by staining for β-casein, which was not present in knockout transplants. The alveolar buds present in the knockout transplants showed a histological appearance indistinguishable from those seen in non-pregnant glands from mature animals. Taken together, these observations indicate that the PRLR expressed by mammary epithelial cells is not required for ductal growth and side branching, these effects being regulated by PRLRs in other tissues, but is essential for lobuloalveolar proliferation and the functional differentiation of mammary epithelial cells during pregnancy.

DISCUSSION

The prolactin receptor knockout mouse has proven very useful in determining the contributions of prolactin to the development and physiology of various systems (Ormandy, 1997a; Lucas, 1998; Clement-Lacroix, 1999); however, the analysis of the mammary phenotype has been complicated by the fact that several reproductive functions affecting mammary gland development are altered in the knockout female mice. Thus, the pattern of estrous cycles is changed, no pseudopregnancy occurs, and the females are infertile. Consequently, the abnormalities observed in mammary development and physiology in knockout female mice may be due either to systemic endocrine effects or to the inability of the mammary epithelium to respond to prolactin stimulation. Here, we have used transplantation of mammary epithelium to resolve these two possibilities.

Our study of knockout mice has revealed that a functional PRLR is essential for ductal development during and after puberty. Close examination of the whole mounts shows that the major ducts appear at the same density in mammary glands of all genotypes, suggesting that dichotomous branching, which is known to be estrogen dependent, is unaffected by the absence of the PRLR and that the morphogenetic defect in mutant mice reflects a failure of ductal side branching. This conclusion is supported by the failure of the ductal trees in knockout glands to increase in complexity with age. This indicates that the epithelial cells participating in dichotomous branching respond to signals different from those that engage in side branching.

We are intrigued by the persistence of TEB-like structures in the mammary glands of knockout females. These structures maintain close contact between the apical epithelial cells and stromal fat cells that is typical of normal TEBs, but unlike normal TEBs, they do not continue to advance through the mammary fat pad, having lost their mitotic cell layers. As knockout females age, most of these TEB-like structures become simple duct ends without a distinctive morphology. These aberrant structures are probably the result of the failure of the terminal end buds to differentiate into alveolar buds under the influence of prolactin and may represent an intermediate structure in which mitogenesis and ductal elongation have been suspended but differentiation into an alveolar bud has not occurred. Similar defects in ductal branching and end-bud differentiation are also seen in mice lacking prolactin (Horseman, 1997) or Stat5a (Liu, 1998).

Alterations in ductal side branching and the failure of the

TEBs to regress in the glands of knockout females are not observed when knockout epithelia are grafted into wild-type fat pads, where normally branched ducts now terminate in alveolar buds. These experiments show that PRLRs expressed outside of the mammary epithelium are responsible. Wild-type 129SV mammary epithelium adopted the less branched morphology of the endogenous Rag1$^{-/-}$ glands following transplant, confirming that systemic effects control this aspect of development, as recently demonstrated (Yant, 1998). Use of *in situ* hybridization analysis does not reveal expression of the PRLR in the mammary stromal cells of the mouse (Bera, 1994) or the rat (Meister, 1992; Ouhtit, 1993; Shirota, 1995). This suggests that prolactin is unlikely to act via the mammary stroma in rodents. Instead, the indirect effects of prolactin on mammary gland development are likely to be traced to its role in governing the endocrine system.

A likely candidate hormone is progesterone, a hormone essential for ductal side branching (Brisken, 1998). Progesterone levels are lower in knockout females than in wild-type mice at estrus (6.8 ng/ml compared to 17.9 ng/ml) (Clement-Lacroix, 1999). We speculate that the absence of side branching in the knockout females is due to reduced ovarian progesterone production in these animals. Interestingly estrogen levels are also lower in knockout females than in wild-type females at estrus (37 pg/ml compared to 53 pg/ml) (Clement-Lacroix, 1999), but ductal bifurcation appears to be normal despite this difference. Thus again the process of ductal bifurcation may be distinct from ductal side branching.

Both prolactin and progesterone are necessary for lobuloalveolar development at pregnancy. Transplantation experiments showed that alveolar growth and differentiation during pregnancy are strictly dependent on the presence of the PRLR in the mammary epithelial cells. The complete absence of lobuloalveolar development in knockout epithelia also indicates that placental lactogens must exert their lactogenic effects directly on the mammary epithelial PRLR and not via putative lactogen receptors.

Comparison of transplanted knockout mammary epithelium to that prepared from progesterone receptor-negative mice indicates that side branching requires the presence of the progesterone receptor but not the prolactin receptor, while both receptors are essential for alveolar development. Our existing work indicates that the progesterone receptor needs to be present in ductal cells near to the alveoli but is not required in the epithelial cells of the alveoli per se (Brisken, 1998). This suggests that progesterone acts to induce paracrine signals that help to initiate or organize alveologenesis. Progesterone and prolactin interact in a number of ways to control alveolar development. First, progesterone increases expression of the prolactin receptor while prolactin increases the expression of the progesterone receptor in mouse mammary gland (Sakai, 1979; Edery, 1985) and human breast cancer cells (Ormandy and Sutherland, 1993; Ormandy, 1997b). This mechanism also operates in the uterus (Chilton, 1988), indicating that this may be a general mechanism allowing a synergistic interaction between these hormones. Recently progesterone has been reported to increase Stat5a gene expression and to induce Stat5a translocation to the nucleus via association with the progesterone receptor (Richer, 1998), providing another mechanistic link between the actions of the two hormones, as Stat5a is a major mediator of PRLR signal transduction, in addition to activation by epidermal growth factor and growth hormone. This interaction may play a part in the progesterone control of ductal side branching, as the ductal side branching defect in Stat5a knockout mice persists when Stat5a knockout mammary epithelium is transplanted to normal hosts (Liu, 1998).

Much of the interaction between prolactin and progesterone remains to be elucidated. We do not know whether the two hormones act on the same target cells, whether they act in synchrony or sequentially, or whether prolactin acts directly on the cells that have been primed by the paracrine signal that was previously released in response to progesterone. Future studies using combined mutations of the progesterone and prolactin receptors may help resolve these issues.

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Estrogen-Dependent Cyclin E-cdk2 Activation through p21 Redistribution

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In order to elucidate the mechanisms by which estrogens and antiestrogens modulate the growth of breast cancer cells, we have characterized the changes induced by estradiol that occur during the G_1 phase of the cell cycle of MCF-7 human mammary carcinoma cells. Addition of estradiol relieves the cell cycle block created by tamoxifen treatment, leading to marked activation of cyclin E-cdk2 complexes and phosphorylation of the retinoblastoma protein within 6 h. Cyclin D1 levels increase significantly while the levels of cyclin E, cdk2, and the p21 and p27 cdk inhibitors are relatively constant. However, the p21 cdk inhibitor shifts from its association with cyclin E-cdk2 to cyclin D1-cdk4, providing an explanation for the observed activation of the cyclin E-cdk2 complexes. These results support the notion that cyclin D1 has an important role in steroid-dependent cell proliferation and that estrogen, by regulating the activities of G_1 cyclin-dependent kinases, can control the proliferation of breast cancer cells.

A variety of models have been proposed to explain how estrogen drives the proliferation of normal mammary epithelial cells and breast cancer cells (8). By acting through the estrogen receptor (ER), estrogen can regulate the transcription of a cohort of responsive genes and in this way appears to regulate cell cycle progression. Even in the face of still-incomplete mechanistic insight into how estrogen regulates growth, effective antitumor therapies directed against the ER have been developed around the use of antiestrogens such as tamoxifen (20). Many studies of the effects of tamoxifen have indicated that tamoxifen acts in a cytostatic fashion on breast cancer cells, causing them to arrest in the G_0/G_1 phases of their growth cycle (36, 49). For these reasons, it is important to understand how estrogens and antiestrogens control G_1 progression.

The central regulator of this process is the cell cycle clock apparatus, which operates in the cell nucleus and is assembled from an array of cyclins and cyclin-dependent kinases (cdks) (45). The activities of the cdks are positively controlled by their association with cyclins and restrained by cdk inhibitors. Included among the latter are p21, p27, and p57, which can inhibit a wide range of cyclin-cdk complexes, and the INK4 family (p15, p16, p18, and p19), which specifically inhibits cdk4 and cdk6 (46).

Extracellular signals such as those conveyed by growth factors affect the activity of cyclins and cdks largely during the G_1 phase of the cell cycle. The most important components of the cell cycle clock apparatus during this period are (i) the D-type cyclins together with their catalytic partners cdk4 and cdk6 and (ii) cyclin E, which interacts with cdk2. Both classes of G_1 cyclin-cdk complexes are known to drive the phosphorylation of the retinoblastoma protein (pRb) (18, 19, 21, 42). This phosphorylation represents a key event in G_1 progression (53). Hypophosphorylated pRb is active in mediating G_1 arrest while hyperphosphorylated pRb appears to be inactive in blocking cell cycle advance.

Overexpression and amplification of G_1 cyclin genes have been observed in a number of primary breast cancers and in tumor-derived cell lines (7, 23, 24). For example, amplification of the chromosomal region 11q13 containing the cyclin D1 gene is frequently observed in breast cancer (26). This amplification seems to occur preferentially in ER-positive tumors and has been linked to poor prognosis (1, 44). Consistent with an important causal role in breast cancer, cyclin D1 overexpression can be observed in primary breast cancers, even at early stages of the disease (3, 54). Microinjection of antibodies or antisense to cyclin D1 during G_1 can prevent cell cycle progression of pRb-positive breast cancer cell lines (3). Moreover, ectopic expression of the cyclin D1 gene in the breast cancer cell line T47D shortens G_1 and induces cell cycle progression (33). Overexpression of cyclin D1 in breast cancer cells also reduces their rate of exiting from the cell cycle, allowing cell cycle progression and pRb phosphorylation even in the absence of growth factors (56).

Other research using mouse models has supported the notion that cyclin D1 plays a central role in regulating the proliferation of mammary epithelial cells. Transgenic mice expressing cyclin D1 under the control of the mouse mammary tumor virus promoter develop mammary hyperplasias and carcinomas in a pregnancy-dependent fashion (51). Moreover, the mammary glands of mice lacking the cyclin D1 gene fail to undergo full development during pregnancy while virtually all other tissues in these mice develop normally (12, 47). Since the main extracellular regulators of mammary development are ovarian steroids, the above results strongly support the notion that cyclin D1 is involved in mediating the steroid-dependent growth of mammary epithelial cells.

Indeed, several reports have suggested a role for steroids in regulating cyclin D1 expression. Musgrove et al. (32) were able to associate progesterone-dependent G_1 progression with changes in the expression of cyclin D1. Work from the same laboratory has suggested that the main target of antiestrogen action is cyclin D1 (52). Treatment of ER-positive breast carcinoma cell lines with antiestrogens led to an increase in hypophosphorylated pRb and to G_1 arrest. Decreases in expression of cyclin D1 preceded the antiestrogen-mediated cell cycle arrest. These changes were followed by a decrease in cdk2 kinase activity (52).

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More recently, direct regulation of cyclin D1 transcription by estrogen has been shown by others (2). In these recent studies, estrogen was able to overcome the cell cycle arrest imposed by the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin by inducing cyclin D1 expression and pRb phosphorylation. The effects of estrogen were attributed to an estrogen-responsive regulatory region between −934 and −136 bp of the human cyclin D1 promoter. The activation of the cyclin D1 promoter by estrogen was independent of mitogen-activated protein kinase activity, which is inhibited by simvastatin (4).

To characterize more precisely the mechanisms by which estradiol induces cell cycle progression, we have studied MCF-7 cells arrested by tamoxifen. We present evidence that release of the cell cycle block by the addition of estrogen leads to rapid activation of cyclin E-cdk2 kinase and pRb phosphorylation. This occurs via a mechanism that is dependent upon induction of cyclin D1 by estrogen and a shift of the p21 cdk inhibitor from cyclin E-cdk2 to cyclin D1-cdk4 or cyclin D1-cdk6.

MATERIALS AND METHODS

Cell culture and synchronization. MCF-7 cells were obtained from M. Brown (Dana-Farber Cancer Institute, Boston, Mass.). They were routinely cultured in Dulbecco's modified essential medium (DMEM; Gibco-BRL) with 5% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.), penicillin (50,000 U/liter), streptomycin sulfate (50,000 μg/liter), and amphotericin B (Fungizone; 125 μg/liter). For tamoxifen synchronization, cells were plated between 5×10^3 and 10×10^3/cm^2. After 48 to 60 h, medium was changed to DMEM (phenol red free) with 5% charcoal-stripped serum (CSS) (HyClone, Logan, Utah), antibiotics, and 1 μM tamoxifen for 48 h (10). Cells were released from the arrest by addition of 500 nM 17β-estradiol or by changing the medium to fresh DMEM (phenol red free) with 5% CSS and 5 nM 17β-estradiol. For controls, cells were either given ethanol or changed to similar medium without estradiol. In some cases, the latter control contained 1 μM tamoxifen.

Cell cycle analysis. MCF-7 cells were plated for thymidine analysis on either 6-, 12-, or 24-well plates. Thymidine incorporation was assessed by labelling synchronized MCF-7 cells with 1 μCi of [*methyl*-^{3}H]thymidine per ml for 30 min. At the indicated time points, cells were washed once with phosphate-buffered saline (PBS) and once with 5% ice-cold trichloroacetic acid. Then, they were incubated in 5% trichloroacetic acid for at least 30 min on ice. After this incubation, cells were washed three times with water and lysed with 0.1 N NaOH. An aliquot of each sample was quantified by liquid scintillation counting.

For fluorescence-activated cell sorter (FACS) analysis, MCF-7 cells were harvested by trypsinization, pelleted gently, and resuspended in 2 ml of PBS. Cells were fixed by the gradual addition of 5 ml of 95% ethanol while being vortexed. After 30 min at room temperature, cells were stored at 4°C. Before processing, cells were collected by centrifugation and stained by addition of 1 ml of a 50-μg/ml propidium iodide solution. RNase A was added to these samples at a final concentration of 100 μg/ml, and the samples were incubated at room temperature for 15 min. Cell cycle analysis was carried out with a Becton Dickinson FACScan flow cytometer.

Preparation of cell extracts. Cell pellets were lysed for 20 min on ice in Nonidet P-40 (NP-40) lysis buffer (PBS [pH 7.2] containing in addition 250 mM NaCl, 2 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol [DTT], 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 50 μg of phenylmethylsulfonyl fluoride per ml, 1 mM NaF, 1 mM orthovanadate, 60 mM β-glycerophosphate). Cell debris was pelleted by centrifugation at 14,000 rpm for 15 min on an Eppendorf centrifuge (Brinkmann) at 4°C. The supernatant was assayed for protein content by Bradford analysis (Bio-Rad) and either used immediately or flash-frozen on dry ice and stored at −70°C.

Antibodies. Monoclonal antibodies against cyclin E and polyclonal antibodies against cdk2, cdk4, cdk6, p21, and p27 with their competing peptides when available were obtained from Santa Cruz Biotechnology, Santa Cruz, Calif. Monoclonal anti-pRb and polyclonal anti-cdk4 were purchased from Pharmingen, San Diego, Calif. Mouse monoclonal antibody against p27 was from Transduction Laboratories, Lexington, Ky. Monoclonal antibodies against p21 (CP-68 and CP-36) were a kind gift from B. Dynlacht, Harvard University, Cambridge, Mass. Monoclonal antibodies against human cyclin D1 and cyclin E were kindly provided by E. Harlow, Massachusetts General Hospital, Charlestown. Polyclonal antibody against p57 was a generous gift from S. Elledge, Baylor College of Medicine, Houston, Tex.

Western blot analysis and immunoprecipitation. Equal amounts of protein were processed for Western blot analysis by either sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel electrophoresis (PAGE) or SDS–12% PAGE. In general, 100 μg of protein per lane was separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore) by standard protocols. The membrane was blocked in PBS with 5% nonfat dry milk for 1 h. Subsequently, it was incubated for 3 h with a dilution of the specific antibody in PBS (2.5% nonfat dry milk, 0.05% Tween). After five washes with PBS (0.1% Tween), the filter was incubated for 1 h with a 1:5,000 dilution of horseradish peroxidase-linked secondary antibody (Jackson Laboratories). Immunodetection was achieved with an enhanced chemiluminescence system (Amersham).

For immunoprecipitations followed by Western blotting, lysates were incubated with the desired antibody cross-linked to beads for 3 h at 4°C with rocking. Beads were pelleted briefly on a microcentrifuge and washed twice with 1 ml of lysis buffer before electrophoresis and transfer to membrane. For immunodepletion, three sequential immunoprecipitations were carried out with each sample. An aliquot equivalent to 100 μg was taken from each supernatant for Western blot analysis to assess remaining proteins. Quantification of blots was done with PDI software (Huntington Station, N.Y.). Images were processed with Adobe Photoshop software and a Lacie Silverscanner II.

Kinase assays. For histone H1 phosphorylation, the amount of lysate immunoprecipitated varied with the specific antibody used in order to ensure that the kinase assay was conducted within the linear range. After immunoprecipitation, the beads were washed twice with lysis buffer and once with kinase assay buffer (20 mM Tris [pH 7.5], 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM DTT). Beads were incubated with 15 μl of kinase mix (kinase buffer containing 10 μM ATP with 2.5 μg of histone H1 [Boehringer Mannheim] and 16 μCi of [γ-^{32}P]ATP per reaction mixture) for 30 min at 30°C and stopped by addition of Laemmli sample buffer. Gels were stained with Coomassie blue, and excised bands were quantified by Cerenkov counting. To determine inhibitory activity present in extracts, equal amounts of protein or amounts as indicated were mixed and incubated at 30°C for 30 min before immunoprecipitation and kinase assay.

For glutathione *S*-transferase (GST)–Rb phosphorylation, we followed the conditions used previously to evaluate cdk4 activity from MCF-7 cells (15). In brief, cell pellets were lysed in Tween buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 10 μg of aprotinin per ml, 5 μg of leupeptin per ml, 50 μg of phenylmethylsulfonyl fluoride per ml, 5 mM NaF, 100 μM orthovanadate) for 30 min followed by centrifugation for 15 min at 4°C. A total of 500 μg of each extract was immunoprecipitated with cdk4 polyclonal antibodies (Pharmingen or Santa Cruz). Beads were washed twice with Tween buffer and three times with Rb-kinase buffer (50 mM HEPES [pH 7.5], 5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF, 100 μM orthovanadate). The reaction was started by addition of 15 μl of Rb-kinase mix (Rb-kinase buffer with 30 μM ATP, 1 μg of GST-Rb [Santa Cruz], and 16 μCi of [γ-^{32}P]ATP per reaction mixture). After 30 min at 30°C, samples were mixed with Laemmli buffer and analyzed by SDS-PAGE.

cdc25A assay. For cdc25A assays, cyclin E-cdk2 complexes were immunoprecipitated with cyclin E antibodies. The immunoprecipitates were washed with cdc25A wash buffer (50 mM Tris [pH 7.9], 5 mM $MgCl_2$, 1 mM DTT) before addition of the reaction mix. The cdc25A reaction mix (50 mM Tris [pH 7.9], 5 mM $MgCl_2$, 10 mM DTT) contained when indicated recombinant GST-cdc25A (generous gift of Michele Pagano, New York University Medical Center, New York, N.Y.) alone or in the presence of 5 mM Na_3VO_4. The cdc25A reaction was carried out at 30°C for 30 min. The reaction was stopped by addition of 1 ml of cold NP-40 lysis buffer and processed for histone H1 kinase assays.

RESULTS

Characterization of estradiol-induced cell cycle reentry. We have used the estrogen-responsive MCF-7 human breast cancer cell line to study the effects of estradiol on the cell cycle. Exponentially growing cultures of MCF-7 cells were arrested in G_0/G_1 by treatment with 1 μM tamoxifen for 48 h in the presence of CSS. Synchronous release of the tamoxifen-arrested cells occurred after removal of tamoxifen and addition of fresh medium containing CSS and 17β-estradiol (5 nM estradiol). The results of a representative experiment are shown in Fig. 1A. Entry into S phase was determined by measuring thymidine incorporation. The peak of DNA synthesis was observed after 22 h of estradiol treatment, at which time the rate of thymidine incorporation increased more than 10-fold over uninduced levels. Tamoxifen-arrested MCF-7 cells that received only fresh medium containing 5% CSS or with 1 μM tamoxifen were not able to enter into S phase. FACS analysis of MCF-7 cells (Fig. 1B) confirmed that the tamoxifen-arrested cells (T = 0 h) were mostly in G_0/G_1 with a low percentage (8%) of cells in S phase. Following addition of fresh medium containing estradiol, over 50% of the cells had begun replicating their DNA by the time the peak of thymidine

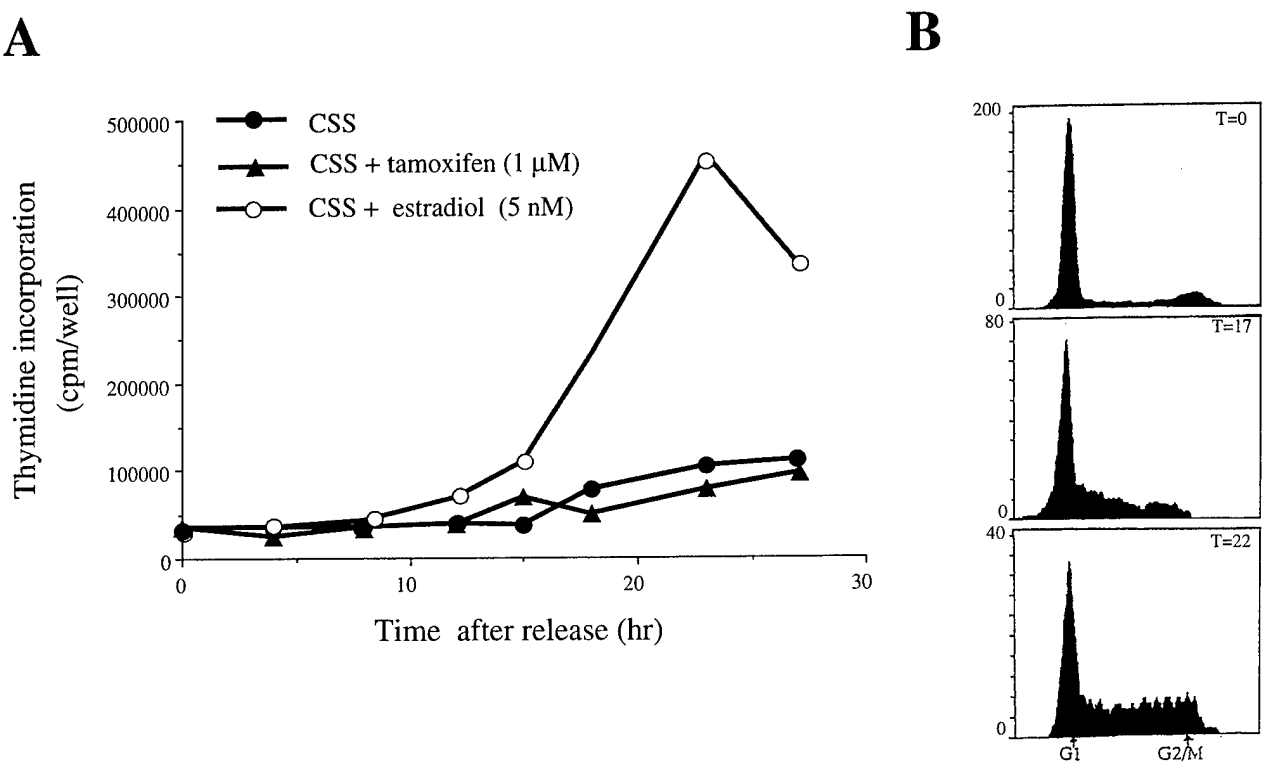

FIG. 1. Estrogen-dependent cell cycle progression. (A) MCF-7 cells were treated with tamoxifen for 48 h in the presence of 5% CSS. At $T = 0$ h, medium was changed to 5% CSS alone or with addition of either 1 μM tamoxifen or 5 nM estradiol. At the times indicated, cells were pulsed with [*methyl*-^{3}H]thymidine for 30 min and thymidine incorporation was determined as described in Materials and Methods. (B) MCF-7 cells treated for 48 h with tamoxifen (0 h) or after change to 5% CSS with 5 nM estradiol for the indicated times (in hours) were fixed, incubated with propidium iodide, and analyzed by FACS. The *y* axes show the number of cells.

incorporation occurred ($T = 22$ h). Synchronized cell cycle reentry of tamoxifen-blocked cells could also be induced by adding relatively high concentrations of estradiol (500 nM) to cells in the continued presence of the tamoxifen blocking agent (data not shown).

Phosphorylation of the Rb protein and escape from tamoxifen inhibition. Previous work addressing the action of tamoxifen on the cell cycle had indicated that MCF-7 cells are sensitive to the antiestrogen tamoxifen and other similarly acting compounds only in a narrow window of time in the cell cycle in early to mid-G_1 (35, 50). However, these studies did not evaluate the effect of tamoxifen on the cell cycle machinery. The period of responsiveness to tamoxifen in G_1 is reminiscent of the effects of transforming growth factor β (TGF-β) on cell cycle advance, which are known to involve primarily a blockage of pRb phosphorylation (14, 25). Thus, after cells have phosphorylated their complement of pRb in late G_1, they become nonresponsive to the growth-inhibitory effects of TGF-β.

For these reasons, we determined whether the acquired refractoriness to tamoxifen in late G_1 could be correlated with phosphorylation of pRb. To do so, we arrested cells with tamoxifen for 48 h and then released them by addition of fresh medium containing 5% CSS and 5 nM estradiol. At various time points thereafter, estradiol-containing medium was removed and replaced with tamoxifen-containing medium. Effects on cell cycle advance were ascertained by measuring the subsequent ability of these cells to incorporate thymidine at $T = 22$ h, the time of peak thymidine incorporation by control cells that had not been treated with tamoxifen following the estradiol-induced cell cycle progression.

As shown in Fig. 2A, most of the cells could be prevented from subsequent S-phase entry if tamoxifen was added back immediately after estradiol addition at $T = 0$ h. However, by 6 h, virtually all the cells were refractory to tamoxifen treatment, achieving levels of thymidine incorporation similar to those of cells that had been exposed continuously to estradiol for 22 h. These data indicated that, within several hours after estradiol addition, tamoxifen (and presumably the ER) no longer exerted control over cell cycle advance.

Western blot analysis of the Rb protein indicated that phosphorylation of pRb correlated closely with the acquisition of refractoriness to tamoxifen (Fig. 2B, top). Densitometric analysis of the different pRb forms revealed the almost complete disappearance of the hypophosphorylated form and its replacement by the hyperphosphorylated form by 6 h. Indeed, the ratio of hypo- to hyperphosphorylated forms dropped dramatically in the first few hours after estradiol stimulation (Fig. 2B, bottom). Surprisingly, the kinetics of pRb phosphorylation preceded by many hours the entry into S phase and differed in this way from the schedule of changes normally seen during cell cycle progression from G_0/G_1 to S phase (6, 9).

Such acquired resistance in mid- to late G_1 to growth inhibition has been observed in a number of other cases besides the aforementioned nonresponsiveness to TGF-β seen in late G_1. Thus, introduction of low levels of cycloheximide or removal of mitogens has been observed to block G_1 advance when applied early in this phase but not in the last several hours of this phase (29, 37, 38). In each case, the time of acquired resistance has been equated with passage through a restriction point (R point). By extension, the acquired refractoriness to tamoxifen inhibition also represents a restriction point transition. As with the other operationally defined R

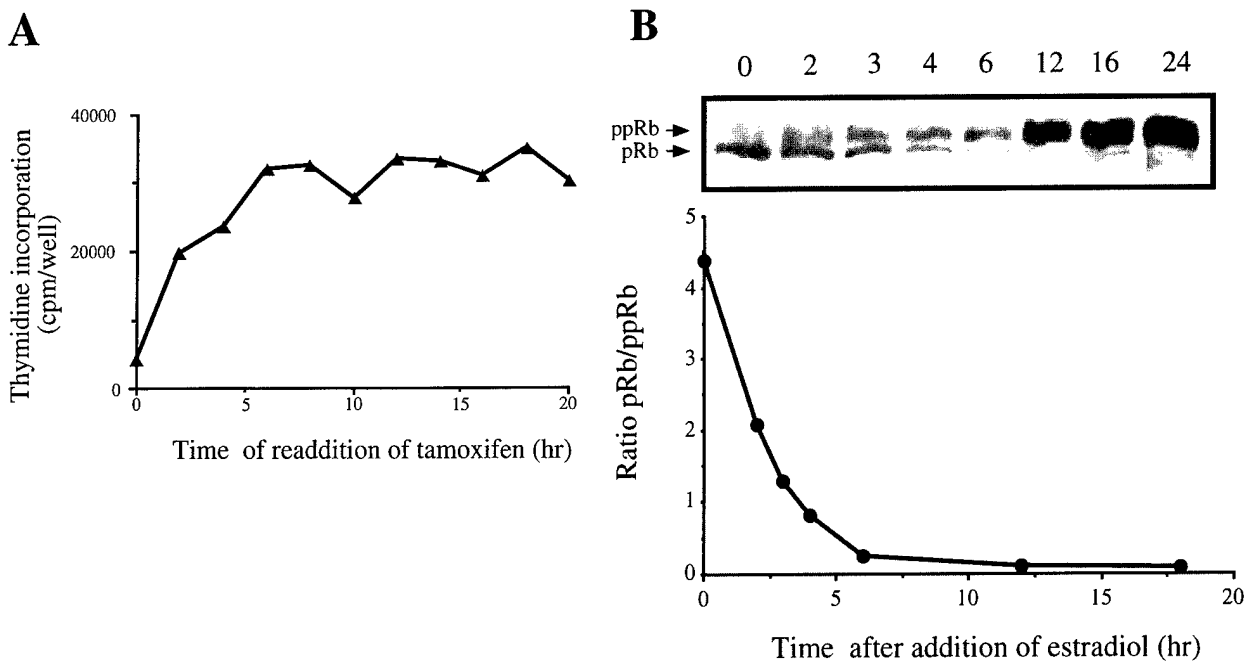

FIG. 2. Refractoriness to tamoxifen and phosphorylation of the retinoblastoma protein (pRb). (A) Tamoxifen-arrested MCF-7 cells were stimulated with 5 nM estradiol at $T = 0$ h. At the indicated times, the medium was replaced with 5% CSS containing 1 μM tamoxifen. After 22 h, all the cells were pulsed with [*methyl*-^{3}H]thymidine and processed as described in Materials and Methods. (B) (Top) Total cell extracts of MCF-7 cells (100 μg) at different time points after release (in hours) from tamoxifen-induced cell cycle block were analyzed for pRb protein by Western blotting. (Bottom) Densitometric analysis of pRb phosphorylation. The ratio of hypophosphorylated pRb to hyperphosphorylated pRb (ppRb) was plotted as a function of time after estradiol addition.

points, this transition is closely contemporaneous with and likely connected with the phosphorylation of pRb.

Regulation of cdks by estradiol in MCF-7 cells. Since pRb phosphorylation is known to be driven largely by cdks, we characterized the effects of estradiol on the activity of the cyclin-cdk complexes implicated in this phosphorylation in mid- to late G_1. Recent reports have suggested that the cyclin D1-cdk4 complex may be a direct target of estrogen action (see Introduction). For this reason, we first characterized the changes in cyclin D1 protein and cyclin D1-cdk4 kinase activity following estradiol addition to tamoxifen-arrested MCF-7 cells. To avoid the possible confounding effects of freshly added serum, we reversed the cell cycle block by adding only estradiol (500 nM final concentration) to tamoxifen-arrested cells. The control cells received only the solvent vehicle (ethanol).

The results shown in Fig. 3A (top) confirmed that estradiol can induce expression of cyclin D1 protein very rapidly. Thus, an increase of cyclin D1 levels was already apparent within 2 h of treatment. Levels of cyclin D1 continued to increase steadily, achieving fivefold higher levels by 6 h. Taken together with the previously reported work (2), the present results indicated a specific effect of estrogen on cyclin D1 levels.

In parallel with these measurements of cyclin D1 levels, we measured cdk4 activity by using GST-Rb as a substrate and antibodies against a peptide derived from the carboxy terminus of cdk4 to immunoprecipitate cyclin D1-cdk4 kinase complexes. We assumed here that the activity of cdk4 could be used as well as an index of the activity of the similarly regulated cdk6 enzyme. As a negative control, we blocked specific binding by preincubating the antibodies with the antigenic peptide. Figure 3A (bottom) shows the changes of cdk4-dependent kinase following estrogen release of tamoxifen-arrested cells. Considerable cdk4 activity toward the GST-Rb substrate was apparent in the tamoxifen-arrested cells prior to their release by estradiol compared to the negative control. After addition of estradiol, this activity increased slowly and steadily, starting at 2 h after addition of estradiol and peaking by 6 h, ultimately reaching threefold higher levels. Similar results were obtained when the kinase assay was performed after immunoprecipitation with cyclin D1-specific antibodies (reference 2 and data not shown). Indeed, we anticipated that we would observe similar kinetics of enzyme activation following immunoprecipitation with either anti-cdk4 or anti-cyclin D1 antibody. While the other D-type cyclins, D2 and D3, are equally able to activate cdk4, our work and that of others have shown that cyclin D2 is not detectable in these cells and cyclin D3 is present in low and constant amounts following estradiol treatment (reference 53 and data not shown).

In addition, we also investigated the status of the other important G_1 cyclin-cdk complex, cyclin E-cdk2. In this instance, cyclin E-cdk2 activity was assayed with immunoprecipitates obtained with an anti-human cyclin E monoclonal antibody and histone H1 as substrate. A dramatic induction of this kinase activity that was first apparent within 2 h of estradiol addition was observed (Fig. 3B, top). At 6 h, this activity had already peaked at a level that was ~20 times higher than that seen at $T = 0$ h (Fig. 3B, bottom). A similar relative induction was seen when cdk2-specific antibodies were used to prepare the precipitates analyzed in the kinase assay (see Fig. 6A). Analysis of cyclin A-dependent kinase activity in the same extracts showed a delayed induction of this enzyme (Fig. 3B, middle) with respect to that of cyclin E with maximum levels reached by 24 h (Fig. 3B, bottom). This induction correlated well with entrance of the cells into S phase.

The changes in cyclin D1-cdk4 kinase could be explained by the increase in cyclin D1 expression. Possible changes in the levels of INK4A were not considered as MCF-7 cells carry a

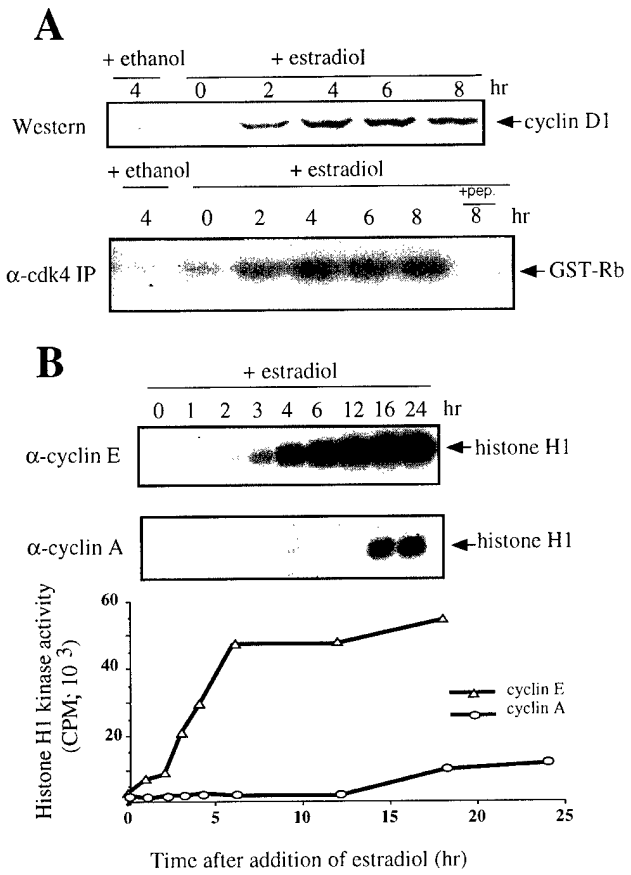

FIG. 3. Effect of estradiol on G_1 cdks. (A) Changes in cyclin D1-cdk4 after estradiol addition. (Top) Western blot analysis of cyclin D1 expression at the indicated time points after the addition of 500 nM estradiol or ethanol to tamoxifen-arrested cells. (Bottom) The same extracts were immunoprecipitated with a polyclonal antibody against the carboxy terminus of cdk4. The kinase activity of these immunocomplexes was measured with GST-Rb as substrate. Background activity (lane +pep.) was determined by blocking cdk4 antibody with a specific antigenic peptide. (B) (Top and middle) Activity of cyclin E (top)- or cyclin A (middle)-associated kinases was assessed with histone H1 as substrate. Tamoxifen-arrested MCF-7 cells were released by a change to 5% CSS with 5 nM estradiol. Cells were harvested and processed for histone H1 kinase assays at the times indicated as described in Materials and Methods. (Bottom) Quantitation of kinase activities. The amount of ^{32}P incorporated into histone H1 in counts per minute was obtained by determining the Cerenkov counts on the excised histone H1 bands.

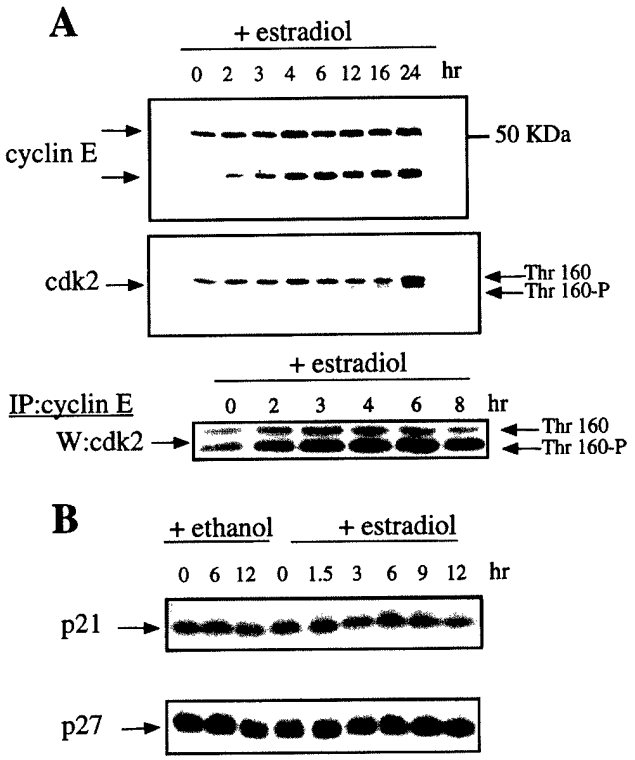

FIG. 4. Effect of estradiol on components of the cyclin E-cdk2 complex. (A) Analysis of cyclin E (top), cdk2 (middle), and cyclin E-associated cdk2 (bottom) from extracts obtained at different time points after release from the tamoxifen block by change to 5% CSS–5 nM estradiol. W, Western blot antibody; IP, immunoprecipitating antibody. (B) Western blot analysis of cell cycle inhibitors, p21 (top) and p27 (bottom), from tamoxifen-arrested cells that received either ethanol or estradiol.

deletion of this gene (31). However, the induction of cyclin E-cdk2 kinase activity still required explanation. Thus, we initiated further experiments to determine how cyclin E-dependent kinase activity was being inhibited by tamoxifen treatment and induced by subsequent estradiol treatment.

Expression of cyclin E, cdk2, and cyclin E-cdk2 inhibitors following estradiol release of tamoxifen-arrested MCF-7 cells. The most likely explanation for the observed changes in the activity of the cyclin E-cdk2 complexes was that estrogen, acting through its receptor, modulated the levels of cyclin E, cdk2, or associated regulatory molecules. Indeed, addition of actinomycin or cycloheximide at the time of estradiol treatment prevented cyclin E-cdk2 activation by estradiol, indicating the need for protein synthesis following estradiol addition (data not shown). However, this need for de novo protein synthesis could not be explained by a requirement for increased levels of cyclin E, since Western blot analysis indicated that the levels of a major form of cyclin E were relatively constant in the 6 h following estradiol addition (Fig. 4A, top). Occasionally, increases in a more rapidly migrating form of cyclin E (22, 23) were apparent during this time period; these increases were not observed reproducibly and could not be correlated with increases in cyclin E-associated kinase activity. The levels of the cdk2 protein also remained essentially unchanged until 24 h (Fig. 4A, middle), long after the functional activation of the cyclin E-cdk2 complexes. A slight increase in the phosphorylated form of cdk2 was seen at 6 h. However, a more remarkable increase in this active form was seen only much later, coinciding with the greatest accumulation of cells in S phase (Fig. 1).

It was also possible that estradiol affected the assembly of cyclin E-cdk2 complexes. To assess this possibility, we analyzed the changes in the levels of the cyclin E-associated cdk2 following estradiol addition to tamoxifen-arrested cells. Cell lysates prepared at different times after estradiol addition were immunoprecipitated with cyclin E-specific antibodies followed by Western blot analysis using anti-cdk2 antibody as probe. This analysis revealed that cyclin E-cdk2 complexes were already present during the tamoxifen arrest and that their levels did not change substantially following estradiol treatment (Fig. 4A, bottom). There was a small increase in the phosphorylated, active cdk2 form following estradiol addition. However, no changes were seen in the cdk-activating kinase activity following treatment with estradiol (data not shown). Together, these data indicated that changes in cyclin E and cdk2 levels or their association with one another could not explain the marked increases in the activity of the cyclin E-cdk2 complexes. This

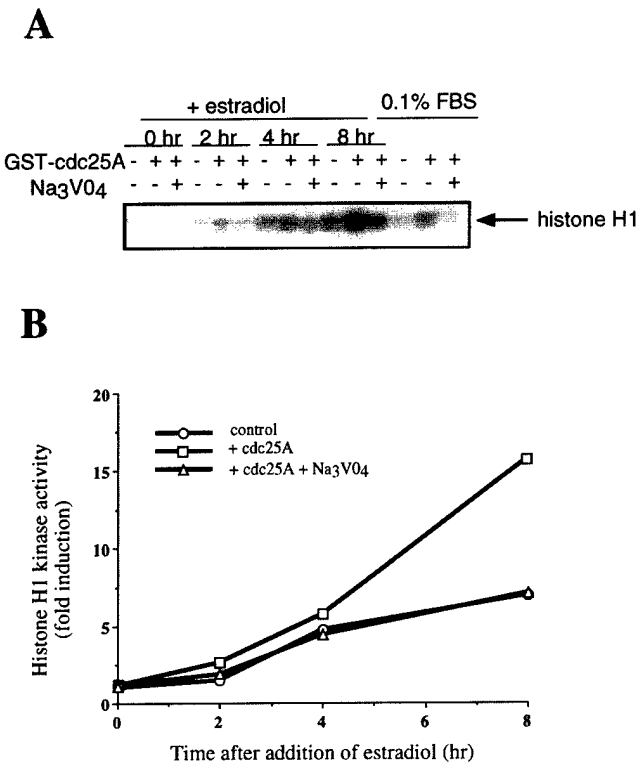

FIG. 5. Effect of recombinant cdc25A on cyclin E-cdk2 activity. (A) Asynchronous MCF-7 cells were treated with either 5% CSS with 1 μM tamoxifen or 0.1% fetal bovine serum (FBS) for 48 h. Tamoxifen-arrested cells were then treated with estradiol for the times shown. Cyclin E-cdk2 complexes were immunoprecipitated from the indicated cell extracts with antibodies specific for cyclin E. The immunocomplexes obtained were split into three and incubated in cdc25A reaction mix in either the presence or the absence of recombinant GST-cdc25A and sodium orthovanadate. (B) Graphic representation of cyclin E-associated histone H1 kinase activity obtained from results shown in panel A.

suggested in turn that other regulators of cyclin-cdk activity were responsible for modulating cyclin E-associated kinase activity following estradiol treatment. More specifically, cdk inhibitors were attractive candidates for such a role. Hence, we determined whether expression of any of the known cdk2 inhibitors was affected by estrogen.

As shown in Fig. 4B, levels of the p21 (top) or p27 (bottom) cdk inhibitors did not change substantially during the hours preceding cyclin E-cdk2 activation. The p57 cdk inhibitor was not detectable in these cells (data not shown). Levels of the INK4 class of inhibitors were not monitored here, as these inhibitors affect only cdk4 and cdk6. Therefore, we concluded that the observed changes in cyclin E-cdk2 activity following estradiol treatment could not be the result of changes in the overall levels of the known cdk2 inhibitors.

Role of cdc25A in cyclin E-cdk2 activation. Cyclin E-cdk2 complexes can also be activated by dephosphorylation mediated by the cdc25A phosphatase (30). Thus, it was possible that the cyclin E-cdk2 complexes from tamoxifen-arrested cells were held inactive by the inhibitory phosphorylation. To address this possibility, we tested the ability of recombinant GST-cdc25A to activate cyclin E-cdk2 complexes immunoprecipitated from cell extracts prepared at different times after estradiol addition. The results shown in Fig. 5A indicated that cdc25A was not able to activate the complexes obtained from tamoxifen-arrested cells. Nevertheless, there was a gradual increase in the ability of cyclin E-cdk2 complexes to be activated by cdc25A in complexes obtained from cells treated with estradiol. This activation was abolished in the presence of the tyrosine phosphatase inhibitor Na_3VO_4 (Fig. 5A). Quantitation of the activation induced by cdc25A indicated that, while cyclin E-cdk2 complexes obtained from tamoxifen-arrested cells were not significantly activated, complexes from cells treated with estradiol for 8 h were activated more than twofold (Fig. 5B). A similar fold activation was also seen when cyclin E-cdk2 complexes obtained from serum-starved MCF-7 cells were used (Fig. 5A, last three lanes). Taken together, these results suggested that the absence of cyclin E-cdk2 kinase activity during tamoxifen-mediated cell cycle arrest was not due to inhibitory phosphorylation of cdk2 attributable in turn to the lack of cdc25A activity.

Analysis of cdk-inhibitory activity in tamoxifen-arrested cells. The absence of substantial changes in the levels or functioning of the various molecules that contribute to cdk2 activity caused us to undertake direct biochemical analysis of cdk2 complexes prepared from cells treated with tamoxifen or estradiol. In particular, prior to immunoprecipitation we mixed extracts from tamoxifen-blocked cells (0 h) with those prepared from cells that had been released from the tamoxifen block by 8 h of estradiol treatment (8 h). In doing this, we hoped to determine whether the tamoxifen-blocked cells contained a soluble inhibitor of cdk2 activity.

The results of these in vitro assays, shown in Fig. 6A, indicated that tamoxifen-arrested cells did indeed contain an activity capable of reducing the activity of cdk2 to levels as low as those observed with extracts from tamoxifen-arrested cells. This inhibition of estradiol-treated extracts was seen when either cyclin E- or cdk2-specific antibodies were used to immunoprecipitate cyclin E-cdk2 complexes prior to assay for kinase activity (Fig. 6A). Moreover, this inhibition was seen only when the extract mixtures were preincubated at 30°C, not when they were preincubated at 4°C (Fig. 6A, last two lanes).

These findings pointed to the presence of a soluble inhibitory substance in tamoxifen-arrested cells capable of abolishing the activity of cyclin E-cdk2 complexes. We assumed tentatively that this inhibitory substance detected in vitro was responsible for the observed inhibition of cyclin E-cdk2 activity in tamoxifen-arrested cells.

Changes in inhibitory activity after estradiol-induced release of the cell cycle block. We wished to monitor the fate of this inhibitory substance following the release of cells from the tamoxifen block. To do so, we tested the inhibitory activity from cells prepared at different time points after estradiol addition. In parallel, we also measured the activity of cdk2-dependent kinase from the same extracts. Figure 6B (left) shows high levels of cyclin E-cdk2 activity at 8 h after addition of estradiol. Mixing of this extract (T = 8 h) with an extract prepared from tamoxifen-arrested cells led to complete inactivation of the induced activity (Fig. 6B, right) in confirmation of the results reported above. The inhibitory activities obtained by mixing cell extracts prepared from cells at various times after estradiol treatment were then compared with the inhibitory activity observed in this 0-h extract (100%). Extracts prepared from cells that had been treated for 2 h with estradiol had only 40% of the inhibitory activity of the tamoxifen-arrested extract, and this activity was reduced further at 4 h, being undetectable by 8 h of estradiol treatment.

These results indicated that estradiol treatment caused the rapid loss of the inhibitory activity that had accumulated during the tamoxifen-imposed cell cycle block. However, the fact that the disappearance of the inhibitory activity did not lead immediately to a reciprocal increase in cdk2 kinase activity suggested that other cdk regulators such as the cdk-activating

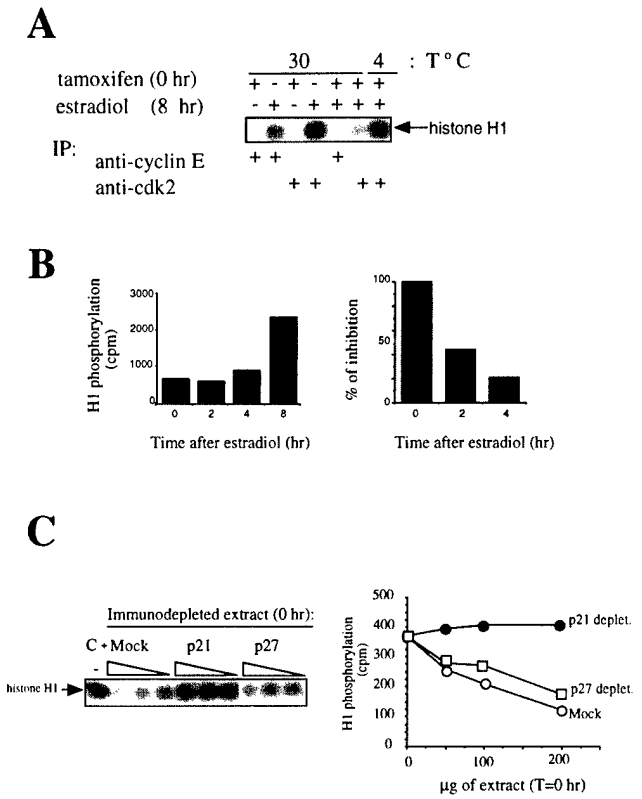

FIG. 6. Characterization of cdk2-inhibitory activity present in tamoxifen-arrested cells. (A) Temperature-dependent inhibition of cyclin E- or cdk2-associated kinase activity. Extracts from tamoxifen-arrested cells and estradiol-stimulated cells alone or mixed as indicated were subjected to immunoprecipitation with cyclin E- or cdk2-specific antibodies. The immunocomplexes were then assayed for kinase activity against histone H1. (B) Changes in cdk2-dependent kinase activity (left) and inhibitory activity (right) after addition of 0.5 μM estradiol to tamoxifen-arrested cells. (C) Immunodepletion of inhibitory activity against cyclin E-cdk2 kinase. (Left) Extracts from tamoxifen-arrested cells were immunodepleted with antibodies against p21 (CP-68) and p27 (SC-528) or irrelevant antibodies (mock). The inhibitory activity remaining after immunodepletion was evaluated by mixing the respective supernatants with extracts from estradiol-treated samples (8 h) as for panel A. The mixture was incubated for 30 min at 30°C followed by a standard immunoprecipitation and kinase assay against histone H1. The use of other antibodies against either cdk inhibitor gave similar results. (Right) Quantitation of the results from immunodepletion (left). The level of histone H1 phosphorylation was plotted against the amount of extract from each immunodepletion that was mixed with 100 μg of extracts from cells at 8 h poststimulation.

kinase may be required to achieve full activation of cyclin E-cdk2.

Depletion of cdk2 inhibitors in tamoxifen-arrested cells. Obvious candidates for the cyclin E-cdk2 inhibitory activity described above were the cdk inhibitors, specifically, p21 and p27. While their overall levels in the cell did not change following estradiol treatment, it was possible that they underwent relocalization in the cell in response to estradiol. Accordingly, we attempted to associate the observed inhibitory activity with specific cdk inhibitors, doing so by treating the inhibitory extract with antibodies reactive with one or another of these proteins in order to deplete these molecules. Following antibody treatment, we tested the remaining supernatants for any inhibitory activity that survived immunodepletion by mixing them with the 8-h extract.

We immunodepleted either p21 or p27 from the tamoxifen-treated (0 h) extract with anti-p21 or anti-p27 specific antibodies, respectively. Immunodepletion was performed by three sequential immunoprecipitations to ensure greater than 99% removal of the inhibitor (see Fig. 7B or Fig. 8). Different amounts of extract (T = 0 h) were used to quantify more precisely any resulting changes in inhibitory activity. As shown in Fig. 6C (left), immunodepletion with anti-p21 antibody efficiently abolished the inhibitory activity of the extract prepared from tamoxifen-arrested cells compared to mock-depleted extracts. In contrast, immunodepletion of p27 led to only a slight reduction of the inhibitory activity of the tamoxifen-treated cell extracts (Fig. 6C, right). These observations suggested that the great bulk of the cyclin E-cdk2 inhibitory activity present in the tamoxifen-treated cell extracts was due to p21 molecules present in these extracts while a minor component was due to p27.

Other evidence indicated that p27 was indeed present in these extracts although not in a configuration that permitted it to inhibit cyclin E-cdk2 activity under our mixing conditions. Thus, when extracts from tamoxifen-treated cells were heated to 100°C prior to assay of cdk2-inhibitory activity, greater than 60% of the inhibitory activity present was due to p27 as judged by immunodepletion with anti-p27 antibodies (data not shown). This also indicated that in tamoxifen-arrested MCF-7 cells the levels of p27 as determined by this functional assay were actually greater than those of p21. Hence, in tamoxifen-treated cell extracts, the great bulk, and perhaps all, of the available, active cdk2-inhibitory substance was derived from p21 molecules. p27 molecules, though present in the extracts, were sequestered in heat-labile complexes from which they could be liberated by brief heating. Moreover, as shown in Fig. 6B, this soluble p21-associated inhibitory activity declined dramatically during the hours following estradiol treatment.

Characterization of complexes between p21 and G_1 cyclin-cdks. As shown above (Fig. 3A), estrogen treatment of the MCF-7 cells causes them to express increased levels of cyclin D1. The resulting cyclin D1-cdk4 complexes might act to bind increasing proportions of the cell's pool of p21 and p27 molecules, thereby abstracting them from cyclin E-cdk2. Indeed, just such a model of cdk inhibitor action has also been proposed to operate in other cell types (46). With this model in mind, we determined the changes in the association of p21 with cyclin D1-cdk4 and cyclin E-cdk2 following estradiol addition with the same extracts analyzed previously to test cdc25A activation.

As expected from the results of earlier experiments, levels of cyclin D1 increased very rapidly, reaching levels fourfold higher than those of control by 6 h (Fig. 7A, top). Moreover, immunoprecipitation of p21 followed by Western blot analysis for cyclin D1 revealed an increase in p21-associated cyclin D1 after addition of estradiol (Fig. 7A, bottom). This increase was first seen within 2 h of estradiol treatment and peaked by 6 h with fourfold higher levels than those that were seen in tamoxifen-blocked cells. Therefore, these results indicated that increases in total levels of cyclin D1 by estradiol correlated in time and magnitude with the changes in levels of p21 associated with cyclin D1. The activation of cyclin E-cdk2 complexes occurred at approximately the same time as these changes, suggesting a possible link between the events (Fig. 5).

The observed increases in p21 associated with cyclin D1 following estradiol treatment were compatible with a model in which D1-cdk4 complexes compete with cyclin E-cdk2 complexes for a limited pool of p21 and abstract increasing amounts of p21 from cyclin E-cdk2 complexes following estradiol treatment. However, the above data did not prove this point, as they did not reveal the proportion of total cellular p21 that was present in these two types of cyclin-cdk complexes at various times after estradiol treatment.

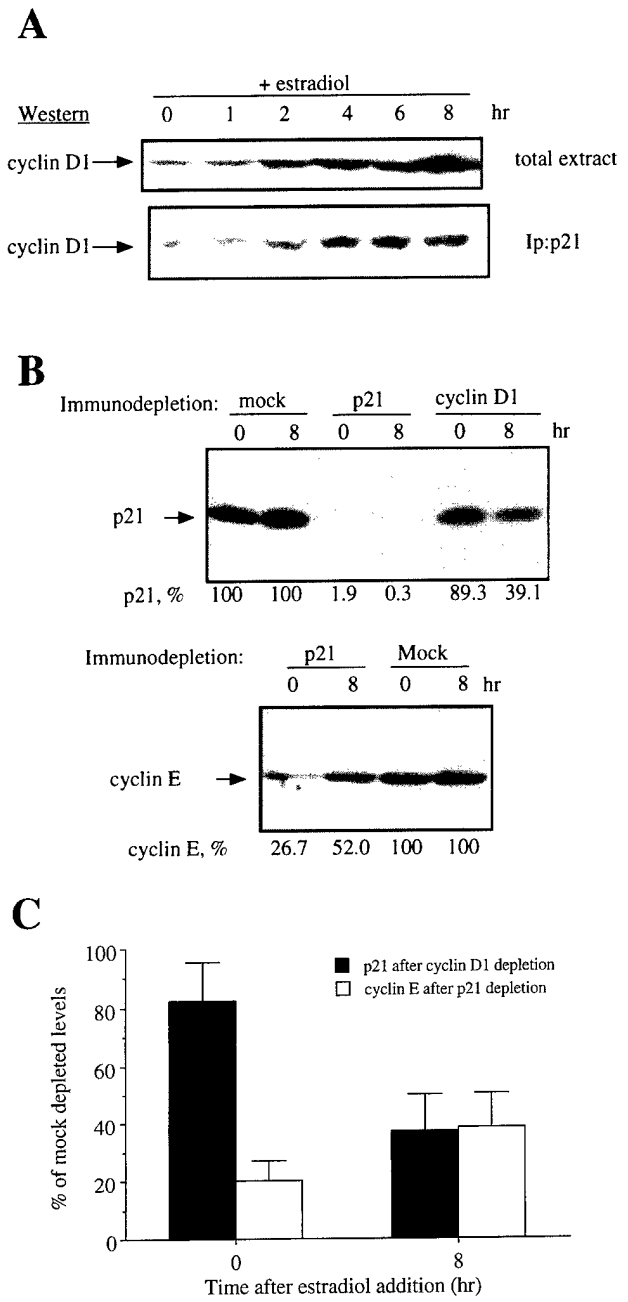

FIG. 7. Changes in p21 distribution among G_1 cyclin-cdk complexes (A). (Top) Cyclin D1 expression from MCF-7 cells after estradiol addition was determined by Western blot analysis of total cell extracts at the time points indicated. (Bottom) Levels of cyclin D1 associated with p21. Cell extracts obtained from tamoxifen-arrested cells or at different times after stimulation with estradiol were subjected to immunoprecipitation with p21 antibodies. The immunoprecipitated complexes were then analyzed for the presence of cyclin D1 by Western blot analysis. (B) (Top) Cell extracts from $T = 0$ and $T = 8$ h after addition of estradiol were immunodepleted by three sequential immunodepletions with nonrelevant antibodies (mock) or with antibodies against either p21 (SC-397) or cyclin D1 (HD-33). The amount of p21 surviving immunodepletion was evaluated by Western blot analysis of 100 μg of total protein with p21-specific antibodies. Numbers at the bottom of each lane represent the amount of specific protein remaining in the supernatant after immunodepletion as determined by densitometric quantitation of images. (Bottom) Levels of cyclin E surviving mock or p21 immunodepletion. The amount of cyclin E remaining in supernatants from mock-depleted extracts was taken as 100%. (C) Quantitation of three independent experiments similar to those described for panel B in which levels of p21 and cyclin E were monitored simultaneously after cyclin D1 and p21 immunodepletion, respectively. Error bars indicate the standard deviations of the samples.

To approach this question directly, we undertook immunodepletion studies with cyclin D1- and p21-specific antibodies. The immunodepleted extracts were then examined for the levels of p21 remaining by Western blot analysis (Fig. 7B, top). Quantitation of p21 levels indicated that p21 immunodepletion removed over 99% of p21 levels from extracts obtained at $T = 0$ h or $T = 8$ h. Anti-cyclin D1 antibodies were similarly successful in removing cyclin D1 from cell extracts (data not shown). When anti-cyclin D1 antibodies were used for immunodepletion, about 10% of the cells' complement of p21 could be removed from extracts prepared at $T = 0$ h while more than 60% of the p21 pool could be immunodepleted from estradiol-treated extracts ($T = 8$ h). Therefore, within 8 h of estradiol treatment, the bulk of cellular p21 associated with cyclin D1-cdk4. We concluded that much and perhaps all of the observed decrease in cdk2-inhibitory activity observed in cell extracts from estradiol-treated cells could be ascribed to this redistribution of p21 to cyclin D1-cdk4 complexes.

To address whether the increase in association of p21 with cyclin D1 coincided with a decrease in the amount of cyclin E complexed with p21, we performed the following experiment. Cell extracts from tamoxifen-arrested cells or from cells treated with estradiol for 8 h were subjected to immunodepletion with p21 antibodies. As shown in Fig. 7B (bottom), the percentage of cyclin E that was not bound by p21 increased from 27% ($T = 0$ h) to 52% after 8 h of estradiol treatment. These results indicated that the activation of cyclin E-cdk2 complexes (Fig. 3B and Fig. 5) correlated with the increase of the amount of cyclin E not complexed to p21. To further strengthen the correlation between the increase in cyclin D1 binding to p21 and the release of p21 from cyclin E-cdk2, we repeated the immunodepletion experiment with several independently obtained samples. The results of three of these experiments are shown in Fig. 7C. The levels of p21 associated with cyclin D1 increased on average from ~20 to ~60% while the levels of cyclin E associated with p21 decreased from ~80 to ~60% following estradiol treatment.

The experiments described above measured the exchange of p21 between cyclin E-cdk2 and cyclin D1 after 8 h of estradiol treatment. However, the induction of cyclin D1 protein levels already occurred between 2 and 4 h after estradiol addition (Fig. 3A and 7A). In order to determine the amount of cyclin E associated with p21 during the period when cyclin D1 expression was induced, we performed an immunodepletion with anti-p21 antibodies at different times poststimulation with estradiol, measuring the amounts of residual cyclin E in cell lysates that survived immunodepletion and were therefore not complexed with p21. An increase in the levels of cyclin E that was not associated in complexes with p21 was evident in lysates prepared from cells at 2 h after estradiol treatment and reached a maximum in lysates prepared after 6 h of estradiol treatment (data not shown). These results correlated with the increase in p21 association with cyclin D1 as shown in Fig. 7A (bottom) and, more importantly, with the increase in cyclin E-cdk2-associated kinase activity (Fig. 5). Taken together, these results indicated that the activation of the cyclin E-cdk2 complex after estradiol stimulation is the result of the redistribution of p21 from cyclin E to cyclin D1.

Characterization of active cyclin E-cdk2 complexes. The data described above suggested a strong correlation between increases in cyclin E-cdk2 complexes devoid of p21 and increases in cyclin E-cdk2 activity. This correlation argued that the pool of cyclin E-cdk2 free of p21 was responsible for the cyclin E-associated histone H1 kinase. However, cyclin E-cdk2 complexes can also be regulated by bound p27 molecules, making it possible that p27 was binding and regulating cyclin

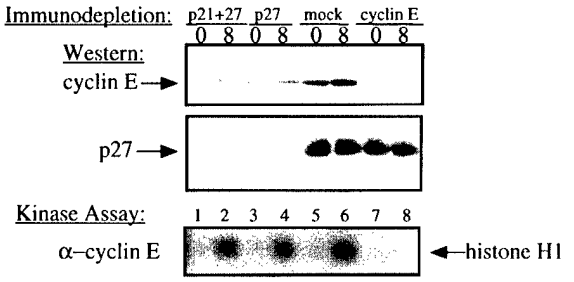

FIG. 8. Characterization of the presence of p27 in cyclin E-cdk2 complexes and formation of active cyclin E-cdk2 complex. Cell extracts from tamoxifen-arrested cells and at 8 h after release by estradiol (times are indicated in hours at the top of the figure) were immunodepleted with nonspecific antibodies (mock) or the indicated specific antibodies. For double immunodepletion of p21 and p27 (p21+p27), a mixture of both specific antibodies was used in the three sequential immunodepletions. (Top) Western blot analysis of cyclin E remaining in the supernatant after immunodepletion. A total of 100 μg of total protein from each supernatant was analyzed. (Middle) Western blot analysis of p27 remaining in the supernatant after immunodepletion. (Bottom) Cyclin E-dependent kinase activity against histone H1 was assayed in supernatants after respective immunodepletions.

E-cdk2 activity in a manner similar to that of p21. For this reason, we determined if the association between p27 and cyclin E-cdk2 complexes was affected by estradiol. Immunodepletions with anti-p27 antibodies were performed on extracts obtained from cells either arrested by tamoxifen or released by estradiol for 8 h with anti-p27 antibodies. p27 antibodies were able to immunodeplete similar levels of cyclin E (>80%) at both time points (Fig. 8, top; compare lanes 3 and 4). In this experiment, p27 was efficiently immunodepleted by the specific antibodies where no effect on p27 was observed in mock-depleted extracts (Fig. 8, middle). To our surprise, either the anti-p21 or the anti-p27 antibody could deplete the majority (over 60%) of cyclin E. One explanation for this apparent discrepancy is that some cyclin E-cdk2 complexes bind p21 and p27 molecules simultaneously. Nonetheless, these results did indicate that changes in p27 association with cyclin E-cdk2 could not explain the observed increases in cyclin E-cdk2 activity following estradiol treatment.

These conclusions were based on the notion that association of cyclin E-cdk2 complexes with either p21 or p27 resulted in the functional inactivation of the cyclin-cdk complex. To validate this directly, we immunodepleted lysates with p21 and p27 antibodies simultaneously. An initial experiment indicated that such immunodepletion was able to completely remove cyclin E (~99%) from cells arrested with tamoxifen (Fig. 8, top, lane 1). The same immunodepletion of p21 and p27 from extracts of estradiol-treated cells containing active cyclin E-cdk2 complexes still immunodepleted the bulk (90%) of cyclin E (lane 2). These results indicated that, even at the peak of activity, the pool of cyclin E-cdk2 complexes free of cdk inhibitors represented only a minor proportion of the total cyclin E-cdk2 complexes present in the MCF-7 cells.

To test if the observed cyclin E-cdk2 activity was associated exclusively with the cyclin E-cdk2 complexes that were free of p21 and p27, we evaluated the cyclin E-dependent kinase activity remaining in the supernatants of the extracts that were previously immunodepleted with p27 antibodies either alone or in conjunction with p21 antibodies (Fig. 8, top). As control, we assayed the cyclin E-dependent kinase activity left in the supernatant fractions after cyclin E immunodepletion. The results of histone H1 kinase assays after immunoprecipitation of these immunodepleted extracts with cyclin E antibodies are shown in Fig. 8 (bottom). As expected, cyclin E immunodepletion completely removed all cyclin E-dependent kinase activity from the extracts (lanes 7 and 8). However, neither p27 immunodepletion nor simultaneous immunodepletion of p21 and p27 decreased the cyclin E-dependent kinase activity of the extracts significantly compared to mock-depleted extracts (lanes 1 to 6). Thus, we concluded that the active cyclin E-cdk2 complexes formed after estradiol-induced cell cycle progression are devoid of these two cdk inhibitors.

Taken together, these results argue that estradiol was able to cause a reduction in the levels of cdk-inhibitory activity associated with cyclin E-cdk2 complexes during the tamoxifen arrest. This reduction was achieved through the induction of cyclin D1 expression, which allowed the formation of cyclin D1-cdk4-cdk6 complexes; these complexes, in turn, served to abstract p21 associated with cyclin E-cdk2, giving rise to active cyclin E-cdk2 complexes.

DISCUSSION

The studies presented here describe the effects that estrogen has on the growth of estrogen-sensitive MCF-7 cells. We conducted these experiments with the aim of determining the specific mechanisms by which estrogen affects the cell cycle clock and leads thereby to the proliferation of mammary epithelial cells. Our data indicate that estrogen can induce rapid and strong activation of cyclin E-cdk2 complexes through its ability to increase cyclin D1 expression. The observed rapid response of the cell cycle clock apparatus to estrogen stimulation argues for a direct effect of estrogen on one or more components of this apparatus. Conversely, it provides evidence against an indirect mechanism involving induction by estrogen of growth factors that in turn act in an autocrine fashion to elicit the observed responses.

The upregulation of cyclin E-cdk2 activity took place as a consequence of the increased cyclin D1 expression induced by estrogen. The increased levels of cyclin D1 led to increased cyclin D1-cdk4 complexes (2, 40), a resulting increase in the amount of the p21 cdk inhibitor associated with this cyclin-cdk complex, and a corresponding reduction in the amounts of p21 bound to cyclin E-cdk2 complexes. This redistribution of the p21 inhibitor then permitted activation of the cyclin E-cdk2 complexes. Similar models have been proposed to explain the mechanism of action of growth inhibitors. For example, TGF-β, by inducing expression of p15 or by downregulating cdk4, can lead to a redistribution of the p27 cdk inhibitor, from cyclin D1-cdk4 to cyclin E-cdk2 complexes (11, 16, 43).

Role of cyclin D1 protein in proliferation of ER-positive cells. Cyclin D1 gene amplification is observed in 15 to 30% of breast cancers (39). A strong correlation of increased levels of cyclin D1 mRNA with ER overexpression has also been noted elsewhere (7). Moreover, this amplification has been associated with a poor prognosis for ER- and progesterone receptor-positive breast tumors (5). Our results indicate that estrogen can induce expression of cyclin D1 in the first hours after its addition to tamoxifen-arrested cells (Fig. 3A and 7A). Thus, these results support the view that estrogen affects expression of cyclin D1 directly.

The model presented here suggests that the ER and cyclin D1 conspire to drive human mammary carcinoma cells through the G_1 phase of their cell cycle. High levels of the ER can cause high expression of cyclin D1 and the resulting removal of p21 from cyclin E-cdk2 complexes. Alternatively, high levels of cyclin D1, which may result from amplification of the cyclin D1 gene or other ER-independent mechanisms, may achieve the same end. Moreover, tumors expressing high levels of either the ER or cyclin D1 may be able to overcome the inhibitory

effects of concomitantly expressed p21 by sequestering the latter in cyclin D1-cdk4 or cyclin D1-cdk6 complexes. Our model is further supported by the work of Musgrove et al. (34). These authors have developed an inducible cyclin D1 system using another ER-positive breast cancer cell line (T47D). Induction of cyclin D1 expression in these cells leads to activation of cyclin E-cdk2 kinase, pRb hyperphosphorylation, and cell cycle progression.

Induction of cyclin D1 by estrogen recruits p21. The present data indicate that the cdk inhibitor p21 serves to couple cyclin D1 levels with the activity of cyclin E-cdk2. We conclude that p21 is involved in the observed cyclin E-cdk2 activation based on the following facts. (i) Extracts obtained from tamoxifen-arrested cells contain a readily detected cdk2-inhibitory activity (Fig. 6A). (ii) This activity can be immunodepleted by p21-specific antibodies but not by anti-p27 antibodies (Fig. 6C). (iii) The ability of p21 antibodies to immunodeplete cyclin E decreases after estradiol treatment of tamoxifen-arrested cells (Fig. 7B, bottom). (iv) Immunodepletion of cyclin D1 removes most of the p21 from extracts of estradiol-stimulated cells but not when extracts from tamoxifen-arrested cells are used (Fig. 7B, top). (v) Levels of cyclin D1 that are associated with p21 increase substantially following estrogen-mediated release of the tamoxifen block (Fig. 7A).

While this work was in progress, Foster and Wimalasena (13), using methionine-glutamine-deprived MCF-7 cells, also observed increased synthesis of cyclin D1, cyclin E-cdk2 activity, and pRb hyperphosphorylation after estradiol-induced cell cycle progression. However, these authors propose that the regulation of cyclin E-cdk2 activity is due to a decrease in p27. The slower kinetics of cyclin E-cdk2 activation and the changes in p27 levels observed by these authors may be due to their use of amino acid starvation to synchronize MCF-7 cells.

Our model of cyclin D1-mediated p21 redistribution is further supported by biochemical measurements that gauge the relative affinities of the p21 cdk inhibitor for association with the cyclin E-cdk2 and cyclin D2-cdk4 complexes. Others have shown elsewhere (17) that the K_i value for inhibition of cyclin E-cdk2 by p21 is 3.7 nM while that for cyclin D2-cdk4 is 0.6 nM. We presume that these inhibitory concentrations reflect the relative affinities of p21 for these two types of complexes and that the affinity of p21 for cyclin D2-cdk4 is similar to that for cyclin D1-cdk4. These relative affinities of p21 would therefore explain the ability of cyclin D1-cdk4 or cyclin D1-cdk6 complexes to abstract p21 from cyclin E-cdk2 complexes. Consistent with this notion is the recent demonstration that cyclin D1 is more effective than cyclin E in rescuing p21-dependent growth suppression (28).

Yet other lines of evidence point to a role played by cyclin D-cdk4 or cyclin D-cdk6 complexes as a reservoir of cdk inhibitors (41, 46, 48). For example, the levels of different cdk inhibitors relative to those of cyclin D-cdk4 or cyclin D-cdk6 complexes may determine a signal threshold level that determines the timing in G_1 of cyclin E-cdk2 activation and consequent pRb phosphorylation. Consistent with this reservoir model, others have shown that p53-mediated growth arrest through induction of p21 can be overcome by ectopically expressed, inactive forms of cdk4 and cdk6 (27).

The dramatic increase of cyclin E-cdk2 catalytic activity following estradiol treatment is due to the translocation of p21 from the cyclin E-cdk2 complexes to cyclin D1-cdk4 complexes. Perhaps surprisingly, this marked increase in the functioning of the cyclin E-cdk2 complexes is attributable to the activity of only a small minority of the complexes present in the estrogen-treated cells. Thus, after estradiol treatment, the entirety of the cyclin E-cdk2 catalytic activity is traceable to a small proportion of the cyclin E-cdk2 complexes that have been freed of p21; the vast majority of these complexes remain associated with cdk inhibitors and in a functionally inactive state. Therefore, in contrast with previous reports (17, 55), we find that the dissociation of p21 from cyclin E-cdk2 complexes is essential for their functional activity. A similar requirement for the removal of p21 from cdk2 complexes has been noted recently, arguing that p21 interaction is strictly inhibitory for cyclin E-cdk2 complexes present in certain cells (27).

In summary, we conclude that estrogen, by regulating cyclin D1 expression and p21 distribution, can control cyclin E-cdk2 activity and pRb phosphorylation in breast cancer-derived MCF-7 cells. This major role of estrogen in controlling levels and activities of G_1 cyclin and the associated kinases fits well with its essential role in driving mammary epithelial cell proliferation.

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Advances in Brief

Functional Activity of Ectopically Expressed Estrogen Receptor Is Not Sufficient for Estrogen-mediated Cyclin D1 Expression[1]

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Abstract

Estrogen receptor function can drive cyclin D1 expression and proliferation in human breast cancer cells (MCF-7). Recent studies showing that estrogen receptor-positive epithelial cells in the human mammary gland are nonproliferative suggest that the direct mitogenic effect of estrogen on mammary epithelial cells may be acquired during breast cancer development. Because estrogen-dependent cyclin D1 expression has been linked to its mitogenicity, we characterized the ability of estrogen to regulate cyclin D1 expression in estrogen receptor-negative breast cancer cells (MDA-MB-231) and nontransformed human keratinocytes (HaCaT) stably expressing the estrogen receptor. In both cases, estrogen receptor function did not induce cyclin D1 expression. Although MCF-7 cells respond to estrogen by inducing the AP-1 family components c-Fos and c-Jun, HaCaT cells expressing estrogen receptor do not. These results may explain the lack of estrogen-dependent cyclin D1 expression and proliferation in cells ectopically expressing the estrogen receptor. Therefore, estrogen receptor function alone is not sufficient for estrogen-dependent cyclin D1 expression and proliferation. Other transcriptional cofactors that allow estrogen receptor to induce expression of AP-1 may be required for estrogen to act as a mitogen.

Introduction

Estrogen is an essential hormone that controls the normal physiology of the mammary gland and breast cancer development. To determine how estrogen regulates growth of breast cancer cells, we and others have characterized the effects of estrogen on the cell cycle of MCF-7 breast cancer cells (1–4). Such studies have indicated that the induction of cyclin D1 by estrogen may be a key to understanding estrogen-dependent proliferation. This estrogen-dependent expression of cyclin D1 is essential for estrogen-induced proliferation of MCF-7 cells (5) and is the earliest estrogen-mediated effect on the cell cycle machinery (3, 4). Ectopic expression of cyclin D1 in MCF-7 cells mimics estrogen effects on the cell cycle (6). In addition, inducible overexpression of cyclin D1 in these cells reverses the growth-inhibitory effects of antiestrogen (7). Together, these results suggest that the ability of estrogen to drive cyclin D1 expression is crucial for the proliferation of ER[3]-positive breast tumors.

The precise molecular mechanism by which estrogen and its receptor control cyclin D1 expression is at present poorly defined. Cyclin D1 does not represent a classical ER target gene, because the cyclin D1 promoter lacks an ERE. Altucci *et al.* (1) mapped the estrogen-responsive region to a fragment between −944 and −136 of the cyclin D1 promoter. Several potential binding sites for known transcription factors can be found in this region of the promoter, such as a site for the AP-1 transcription factor. This suggested the possibility that estrogen regulates cyclin D1 expression through modulation of AP-1 activity. However, no conclusive evidence exists at present to validate this hypothesis. It is unclear whether the presence of ER is sufficient to confer estrogen-mediated cyclin D1 expression.

Several investigators have stably introduced ER into ER-negative cells and have demonstrated estrogen-dependent expression of classical ERE-containing genes such as *cathepsin D* and *transforming growth factor-α* (8–10). However, estrogen did not stimulate proliferation of these cells, and in many cases, estrogen caused growth inhibition (10, 11). In addition, a recent histopathological study demonstrated that normal ER-positive cells in the human mammary gland *in vivo* are nonproliferating, whereas ER-positive breast cancer cells are actively proliferating (12). These two observations together suggest that the presence of ER *per se* is not sufficient for estrogen-induced proliferation of the ER-positive cell.

A possible explanation for the lack of estrogen-stimulated proliferation in cells ectopically expressing ER is that estrogen is unable to induce cyclin D1 expression in these cells. To test this hypothesis, we determined whether MDA-MB-231 and HaCaT cells engineered to express ER can up-regulate cyclin D1 in response to estrogen. Our results suggest that expression of ER alone is not sufficient to confer estrogen-inducible cyclin D1 expression. The absence of cyclin D1 induction by estrogen and the ER may be related to the inability of this receptor to regulate the expression of components of the AP-1 transcription factor complex.

Materials and Methods

Cell Culture, DNA, and Transfection. S30 cells were generously provided by Dr. V. Craig Jordan (Northwestern University Medical School, Chicago, IL). The plasmid pCMV-ER was a gift of Dr. Myles Brown (Dana-Farber Cancer Institute, Boston, MA). The ERE-driven luciferase constructs ERE-SV40-luc and ERE2–109-A3-luc were kindly given to us by Dr. Barry Gehm (Northwestern University Medical School, Chicago, IL). ER-expressing clones were obtained after calcium phosphate-mediated transfection of MDA-MB-231 and HaCaT cells with pCMV-ER and selection with G-418. Transient transfections of HaCaT cells were performed using Tfx-50 reagent (Promega Corp., Madison, WI), following the manufacturer's guidelines.

Antibodies and Western Analysis. Preparation of cell extracts and Western blot analysis was carried out as described previously (3). Monoclonal antibody against cyclin D1 (HD-45) was a gift from Ed Harlow (Massachusetts General Hospital, Charlestown, MA). Rabbit polyclonal antibodies used to detect ER (SC-543), c-Fos (SC-52), and c-Jun (SC-44) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Results

Cyclin D1 Regulation in ER-containing MDA-MB-231 Cells. Several studies have shown that estrogen responsiveness of target genes can be obtained by stably expressing ER in ER-negative cells (8–10). However, estrogen does not have the ability to induce proliferation of these ER-containing cells. We wanted to understand this

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[3] The abbreviations used are: ER, estrogen receptor; ERE, estrogen receptor element, CSS, charcoal-stripped fetal bovine serum; FBS, fetal bovine serum.

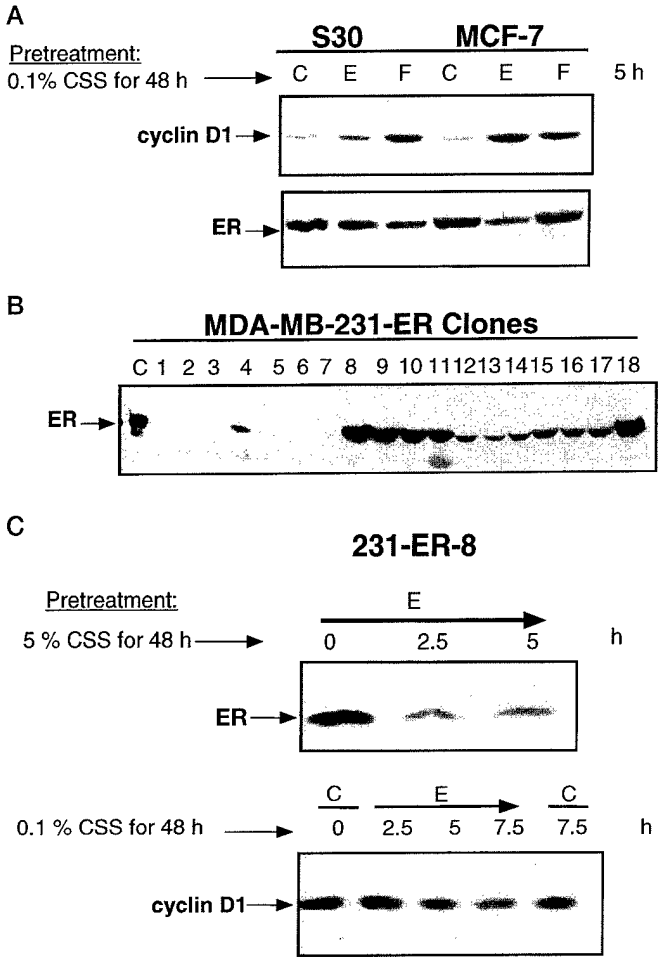

Fig. 1. Characterization of 231-ER cells. *A*, S30 cells and control MCF-7 cells were estrogen and serum deprived by incubating them in phenol red-free medium containing 0.1% CSS for 48 h. After this period, cells were treated with 5 nM of 17β-estradiol (*E*), 5% FBS (*F*), or remained untreated (*C*). Five h later, all samples were harvested and processed for Western blot analysis. *Top*, analysis of cyclin D1 expression. *Bottom*, detection of ER expression. *B*, G418-resistant clones were isolated after transfection of MDA-MB-231 cells with pCMV-ER. Cell extracts were prepared and analyzed for ER expression by Western blot analysis. *C: top*, ER expression after estrogen treatment of steroid-deprived 231-ER-8 cells; *bottom*, cyclin D1 expression after addition of 5 nM 17β-estradiol to 231-ER-8 cells maintained in 0.1% CSS for 48 h.

phenomenon in greater detail by determining whether the lack of proliferation was attributable to the inability of estrogen to induce cyclin D1. For this purpose, we used S30 cells, a derivative of MDA-MB-231, an ER-negative breast cancer cell line, which stably express ER and show estrogen-dependent expression of the progesterone receptor (10).

We wished to determine the ability of the ectopically expressed ER to induce cyclin D1 protein in the ER-expressing S30 cells. For this analysis, we first reduced the basal level of cyclin D1 protein in S30 cells by culturing these cells in phenol red-free medium containing 0.1% CSS. In this way, we also minimized exposure to residual ER-activating agents. After 48 h of serum and estrogen deprivation, S30 cells and MCF-7 cells received either 5 nM 17β-estradiol or 5% FBS as a positive control. Another set of dishes remained untreated to determine the basal levels of cyclin D1. All of the samples were harvested after 5 h of treatment and processed for Western blot analysis.

Estrogen was unable to induce cyclin D1 expression significantly, whereas exposure of these cells to FBS resulted in strong induction of cyclin D1 (Fig. 1*A*, *top*). In contrast, in MCF-7 cells, both estrogen and FBS were capable of strongly inducing cyclin D1 expression (Fig. 1*A*, *top*). This suggested that the presence of the ER in mammary carcinoma cells, although necessary for the estrogen-mediated stimulation of cyclin D1 synthesis in MCF-7 cells, was not sufficient in other cell types.

To confirm that the ectopically expressed ER was functional in our assay, the same Western blot was analyzed for changes in ER protein levels after estrogen treatment. Estrogen can down-regulate expression of its own receptor in MCF-7 cells or in other cells when ER is ectopically expressed (13, 14). This decrease in ER occurs at the protein and mRNA levels and depends on a functional ER. Indeed, addition of estrogen to serum-starved MCF-7 cells caused down-regulation of ER protein expression (Fig. 1*A*, *bottom*), as has been reported previously (13). In a similar way, S30 cells were able to modulate ER expression after estrogen addition (Fig. 1*A*, *bottom*), indicating that the ER is functional in these experiments.

To rule out the possibility that the results obtained were specific to the S30 cell clone, we generated a new series of ER-expressing MDA-MB-231 cells. Eighteen cell clones were isolated after transfection of MDA-MB-231 with pCMV-ER. Twelve of the clones obtained, termed 231-ER, expressed detectable levels of ER as assayed by Western immunoblot analysis (Fig. 1*B*). MCF-7 cells (*left lane*) were used as positive control. Four clones that displayed different levels of ER were further characterized to assess ER function and estrogen-dependent cyclin D1 expression. The results obtained with one of these clones (231-ER-8) are shown in Fig. 1*C*. Expression of ER protein was down-regulated to 30% of the initial level after 2.5 h of estrogen addition to estrogen-deprived 231-ER-8 cells (Fig. 1*C*, *top*). This indicated that ER was functional in these cells. However, cyclin D1 expression was not affected when estrogen was given to serum-starved 231-ER-8 cells (Fig. 1*C*, *bottom*). The other three clones analyzed were also not able to induce cyclin D1 expression after treatment with estrogen (data not shown). We concluded that the inability of estrogen to up-regulate cyclin D1 levels may be due to some specific features of these breast cancer cells (MDA-MB-231) that are extraneous to the ER itself.

Cyclin D1 Regulation in ER-containing HaCaT Cells. One explanation for the inability of the ectopic ER to regulate cyclin D1 expression in the MDA-MB-231 cells is that these cells are derived from tumors, and that some of the changes that they have undergone during tumor progression prevent the ER from interacting functionally with the cyclin D1 promoter. To address this possibility, we expressed the ER in a quite distinct type of human epithelial cell, the HaCaT nontransformed human keratinocyte cell.

Thirty-eight HaCaT clones stably transfected with the pCMV-ER vector were obtained and analyzed for expression of ER. Twelve of these clones expressed ER at levels comparable with those seen in MCF-7 cells. Eight of them, termed HaCaT-ER clones, expressed functional ER as determined by the ability of estrogen to down-regulate its own receptor (data not shown).

To obtain a more quantitative estimate of ER function in these HaCaT-ER cells, we also determined their ability to mediate estrogen-dependent transcription. For this purpose, we measured estrogen-dependent luciferase expression after transient transfection with two distinct promoter constructs that contained either one (ERE-SV40-luc) or two copies (ERE2–109-A3-luc) of the ERE (15). Fig. 2*A* shows the results obtained with the cell clone (HaCaT-ER-38) that gave consistently the highest estrogen-dependent transcriptional induction. The amount of induction obtained (5-fold with ERE-SV40-luc and 10-fold with ERE2–109-A3-luc) is comparable with the response seen with MCF-7 cells (data not shown), thereby confirming the presence of functional ER in HaCaT-ER-38 cells.

We then proceeded to evaluate the ability of the ectopically ex-

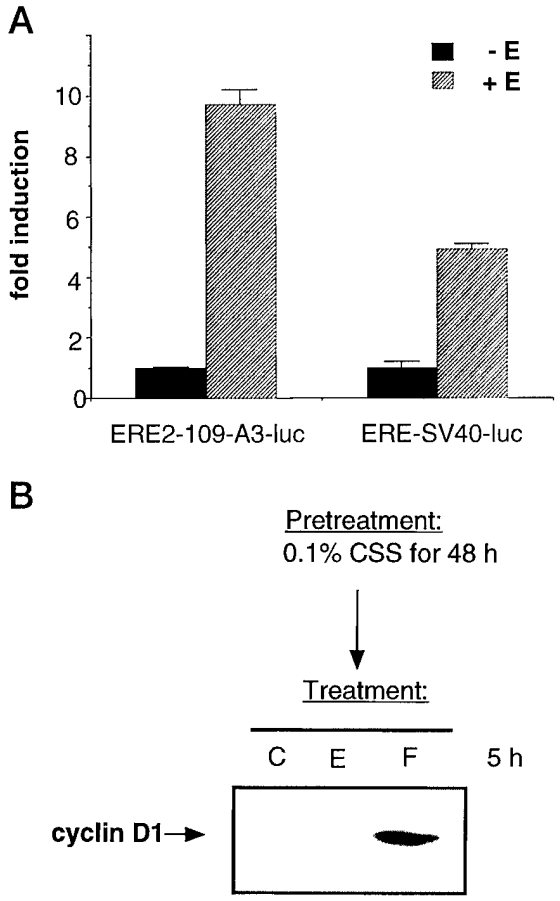

Fig. 2. Analysis of HaCaT-ER-38 cells. *A*, ER function was assessed by transient transfection of HaCaT-ER-38 cells with ERE-driven luciferase constructs. The amount of relative luciferase units obtained in the absence of estrogen was taken as one. *B*, cyclin D1 regulation in HaCaT-ER-38 cells. Asynchronously growing HaCaT-ER-38 cells were placed 0.1% CSS. After 48 h, cells were either treated with 5 nM 17β-estradiol (*E*) or 5% FBS (*F*). Control cells (*C*) received no treatment. All samples were harvested after 5 h, and cyclin D1 expression was determined by Western analysis.

pressed ER in HaCaT-ER-38 cells to activate *cyclin D1* gene expression. To do so, we starved these cells for 48 h to reduce the basal level of cyclin D1 expression. Similar to what we observed with 231-ER cells, estrogen addition was unable to up-regulate cyclin D1 expression, whereas 5% FBS was able to induce cyclin D1 levels dramatically (Fig. 2*B*). Comparable analyses were carried out with eight other ER-expressing HaCaT cell clones, and none of these was able to up-regulate cyclin D1 expression in response to added estrogen (data not shown). Thus, as was seen previously with the MDA-MB-231 cells, the presence of a functional ER did not suffice to allow estrogen to induce expression of cyclin D1.

Changes in AP-1 Components after Estrogen Treatment. These data suggested that the regulation of cyclin D1 expression by the ER is complex and may involve the mediation of other proteins that convey signals between the ER and the cyclin D1 promoter. Such proteins might hypothetically be absent or functionally inactive in the MDA-MB-231 cells and the HaCaT cells, explaining the inability of the ER to activate cyclin D1 expression in these cells.

As mentioned earlier, an attractive candidate for regulating cyclin D1 expression is the AP-1 transcription factor. Estrogen can affect AP-1 either by regulating the synthesis of AP-1 family members (16–18) or by modulating its transcriptional activity (19). Moreover, both the Fos and Jun proteins, two common component subunits of the AP-1 factor, are known to be involved in the regulation of *cyclin D1* gene expression (20, 21). For these reasons, we decided to evaluate whether there was a differential regulation of these genes by estrogen in MCF-7 and HaCaT-ER cells.

To approach this question, we tested whether estrogen could modulate expression of c-Fos and c-Jun levels in either MCF-7 or HaCaT-ER-38 cells. Asynchronously growing MCF-7 and HaCaT-ER-38 cells were serum-starved in 0.1% CSS for 48 h. Cells were treated with either 5 nM 17β-estradiol or 5% FBS and harvested for Western analysis at 1 or 3 h. To begin, we determined the expression of the cyclin D1 protein. Cyclin D1 levels were induced in MCF-7 cells after 3 h of either estrogen or FBS treatment. As before, we observed that although estrogen and serum each were able to induce cyclin D1 expression in MCF-7 cells, only serum succeeded in doing so in HaCaT-ER-38 cells (Fig. 3, *top*).

The same Western blot used to detect cyclin D1 above was subsequently probed for the presence of Fos. The antibody used by us is specific for c-Fos and does not detect FosB, Fra-1, or Fra-2. Because of changes in phosphorylation, c-Fos is present as several distinctly migrating electrophoretic species (22). In MCF-7 cells, the levels of most of these isoforms increased by 3–4-fold after addition of either estrogen or FBS (Fig. 3, *middle*). HaCaT-ER-38 cells showed high basal levels of Fos expression when compared with MCF-7, and no induction of Fos occurred after addition of estrogen. However, FBS was able to augment Fos expression in these cells, suggesting that the lack of response to estrogen is not due to the presence of a c-*fos* gene in these cells that is refractory to further induction. Thus, the inability of the ER to induce cyclin D1 in HaCaT-ER-38 cells was paralleled by its inability to induce Fos synthesis.

A very similar outcome was noticed when c-Jun expression was evaluated (Fig. 3, *bottom*). This molecule is also subject to modification and can be seen as a series of bands. In the absence of serum, MCF-7 cells expressed low levels of the Jun protein. However, addition of estrogen or FBS to serum-starved MCF-7 cells caused a transient induction of Jun protein that parallels the changes detected for Fos. Again, as was the case with Fos, HaCaT-ER-38 cells had high basal levels of Jun that could be superinduced by FBS but not by estrogen. If anything, estrogen caused a decrease in Jun expression. Thus, the ability of either estrogen or FBS to induce cyclin D1 correlated well with their ability to increase Fos and Jun expression. Because induction of AP-1 members occurs within 1 h of addition of estrogen, before any cyclin D1 is observed, this suggests that induction of these genes anticipates and may be required for the proper regulation of cyclin D1 synthesis. Together, these results suggest that the lack of cyclin D1 induction by ectopically expressed ER may be explained by its inability to modulate expression of the c-*fos* and c-*jun* genes.

Discussion

An elucidation of the molecular mechanism of cyclin D1 regulation by the ER is of central importance to our understanding of the molecular pathogenesis of human breast cancers. If estrogen-dependent cyclin D1 expression is necessary for tumor growth, antiestrogens may be able to inhibit cancer growth by preventing estrogen-mediated cyclin D1 expression. Thus, development of antiestrogen resistance may be associated with changes in hormonal regulation of cyclin D1. It is plausible that alterations in the expression or function of specific molecules will allow cyclin D1 expression in the presence of tamoxifen. It is also possible that the ability of estrogen to induce cyclin D1 and proliferation is acquired as a pathological trait during the course of breast cancer development. For this reason, it is important to determine the mechanisms by which cancer cells display ER-dependent cyclin D1 transcription.

In the studies presented here, we have investigated the ability of ER

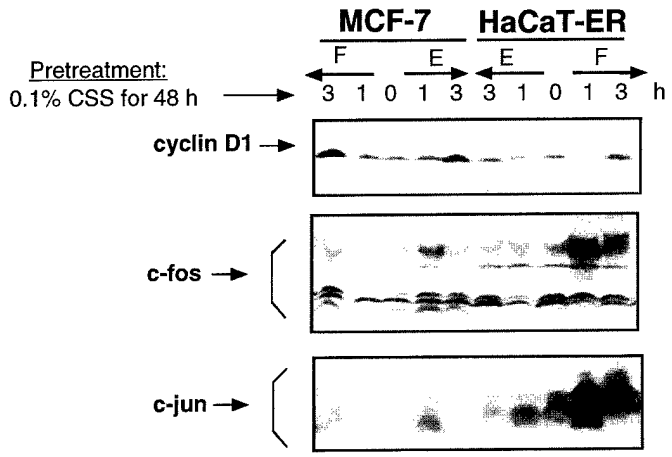

Fig. 3. Expression of AP-1 members after estrogen treatment of MCF-7 and HaCaT-ER cells. MCF-7 and HaCaT-ER-38 cells were estrogen and serum deprived by incubating them in 0.1% CSS for 48 h. At $T = 0$ h, cells received either 5 nM 17β-estradiol (*E*) or 5% FBS (*F*). Samples were harvested at the indicated times and processed for Western analysis of cyclin D1 (*top*), c-Fos (*middle*), or c-Jun (*bottom*).

to drive expression of cyclin D1 after its stable introduction into two distinct, previously ER-negative human cell types. Our aim was to evaluate whether the inability of ectopically expressed ER to confer estrogen-dependent mitogenesis was related to the lack of cyclin D1 induction in ER-negative cells forced to express ER. Our findings indicate that in all of the clones ectopically expressing ER, cyclin D1 expression cannot be augmented by addition of estrogen. Nevertheless, these clones were competent to down-regulate ER expression or drive transcription of an ERE-luciferase construct after addition of estrogen. Moreover, we found that the inability of ER to drive cyclin D1 may be related to the lack of induction of AP-1 components by estrogen. Consequently, these data support the idea that estrogen may control proliferation of breast cancer cells by its ability to induce AP-1 members and cyclin D1.

Cyclin D1 as a Mediator of ER-stimulated Proliferation. The primary cell cycle target of estrogen in MCF-7 cells appears to be cyclin D1. This is supported by the fact that inducible expression of cyclin D1 overcomes growth-arrest mediated by antiestrogens (7). More recently, an extension of this earlier work demonstrated that high levels of cyclin D1 expression led to p21 redistribution, cyclin E-cdk2 activation, and retinoblastoma hyperphosphorylation of the pRB, the retinoblastoma protein, in antiestrogen-arrested MCF-7 cells (6). Thus, the increases of cyclin D1 protein by estrogen may be necessary and sufficient for proliferation of breast cancer cells.

In breast cancer tumors, the expression of cyclin D1 has been correlated with the expression of ER (23). The amplification of cyclin D1 occurs preferentially in ER-positive tumors, and the levels of cyclin D1 parallel in many cases the levels of ER present in the tumors (23). These observations suggest that the functional connections between the ER and cyclin D1 observed in MCF-7 cells *in vitro* correctly model what is seen in breast carcinomas *in vivo*. If this is the case, why does ER expression in ER-negative cells not confer estrogen sensitivity to the *cyclin D1* gene?

In many cases where ER has been stably introduced into ER-negative cells, different endogenous genes can be turned on, depending on the cell type used. One recent report, which appeared while our studies were ongoing, analyzed cyclin D1 expression in cells engineered to ectopically express ER (24). These authors analyzed cell proliferation and cyclin D1 after estrogen treatment of ER-transfected MCF-10AE^{wt5}. Similar to our results, they could not detect any increases in cyclin D1 expression after estrogen treatment of the transfected cells. They did not determine whether ER was transcriptionally active in MCF-10AE^{wt5} cells. However, they found that ER from MCF-10AE^{wt5} had altered ligand-binding affinity when compared with ER derived from MCF-7 cells by sucrose gradient sedimentation.

One reason for the inability of estrogen to drive cyclin D1 in ER-transfected cells could be that the *cyclin D1* locus is not responsive to transcriptional activation in these cells. However, the ability of FBS to rapidly yield increases in cyclin D1 levels argues against this possibility. Thus, the lack of estrogen-mediated cyclin D1 expression is most likely due to the presence or absence of other factors that mediate estrogen-dependent cyclin D1 transcription.

The recent reports that normal estrogen receptor-positive human mammary epithelial cells are nonproliferative suggest that estrogen is unlikely to act as a direct mitogen of normal ER-positive cells in the mammary gland (12, 25). Accordingly, the ability of estrogen to act as a direct mitogen and induce expression of cyclin D1 would appear to represent a pathological aberration acquired during the process of breast cancer progression. This aberration in signaling may occur early during tumor progression, because overexpression of cyclin D1 mRNA has been observed at early stages of breast cancer (26).

AP-1 Members as Potential Mediators of Estrogen-dependent Cyclin D1 Expression. In the search for possible molecular intermediaries between estrogen and cyclin D1 transcription, components of the AP-1 transcription factors emerge as highly attractive candidates. Studies of cells from mice bearing germ-line inactivations of the c-*jun*, c-*fos*, or c-*fosB* genes have revealed that c-Jun and either c-Fos or FosB are necessary for normal transcription of cyclin D1 (20, 21). Moreover, previous mapping of an estrogen-responsive region in the cyclin D1 promoter identified a region of the promoter that contained an AP-1 site but no ERE elements (1). Together, these observations suggest that the ability of estrogen to modulate AP-1 activity may be required for cyclin D1 expression and therefore proliferation.

Several possible scenarios can be considered to explain how estrogen interacts with AP-1: (*a*) the ER protein, like other steroid receptors, may bind physically to the AP-1 factor to activate transcription from AP-1 sites; or (*b*) alternatively, estrogen may act indirectly to drive synthesis of the component subunits of the AP-1 factor, which then proceed to assemble and drive the transcriptional activation of the *cyclin D1* gene locus (27); or (*c*) a third scenario could be that both mechanisms may operate in MCF-7 cells. Our results revealed that there is a differential regulation by estrogen in the synthesis of AP-1 members between MCF-7 cells and HaCaT-ER cells. There is a clear induction of c-Jun and c-Fos by estrogen in MCF-7 but not in HaCaT-ER. However, both gene products are induced by FBS, indicating that, as was the case with the cyclin D1 promoter, the defect in estrogen signaling is not due to promoter silencing. This suggests that the lack of cyclin D1 induction by estrogen may be traced to the inability of estrogen to modulate c-Jun and c-Fos expression in HaCaT-ER cells.

The mechanisms by which estrogen promotes synthesis of AP-1 components in MCF-7 cells are not clear. Several investigators have tried to map estrogen-inducible sequences in the c-*fos* gene (28–30) or c-*jun* gene (31). These studies have led to the identification of a variety of candidate regulatory sequences in the promoters of these genes. For example, a recent study using MCF-7 was able to map the estrogen-responsive region to an imperfect Sp1-binding site (28). The authors claim that induction of c-*fos* expression depends on the formation of a transcriptionally active ER/Sp1 complex. Thus, the inability of estrogen to drive cyclin D1 expression in HaCaT-ER cells may be linked to a deficient interaction between ER and Sp1 in these cells.

In summary, we propose that the ability of ER to drive ERE-

mediated transcription can be dissociated from its role as a mitogen. The competence of estrogen to drive proliferation may be linked to the regulation of cyclin D1 transcription, which in turn may be mediated by the actions of the AP-1 transcription factor. This points to the importance of determining how estrogen modulates AP-1 function and what the specific changes in breast cancer cells are that allow estrogen to affect AP-1, cyclin D1, and proliferation. Understanding these mechanisms may provide clues on how ER-positive breast tumors develop and how they become refractory to antiestrogen treatment.

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Developmental Biology

A paracrine role for the epithelial progesterone receptor in mammary gland development

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ABSTRACT **Recently generated progesterone receptor (PR)-negative ($PR^{-/-}$) mice provide an excellent model for dissecting the role of progesterone in the development of the mammary gland during puberty and pregnancy. However, the full extent of the mammary gland defect in these mice caused by the absence of the PR cannot be assessed, because $PR^{-/-}$ mice do not exhibit estrous cycles and fail to become pregnant. To circumvent this difficulty, we have transplanted $PR^{-/-}$ breasts into wild-type mice, and we have demonstrated that the development of the mammary gland in the absence of the PR is arrested at the stage of the simple ductal system found in the young virgin mouse. Mammary transplants lacking the PR in the stromal compartment give rise to normal alveolar growth, whereas transplants containing $PR^{-/-}$ epithelium conserve the abnormal phenotype. Chimeric epithelia in which $PR^{-/-}$ cells are in close vicinity to PR wild-type cells go through complete alveolar development to which the $PR^{-/-}$ cells contribute. Together, these results indicate that progesterone acts by a paracrine mechanism on a subset of mammary epithelial cells to allow for alveolar growth and that expression of the PR is not required in all the cells of the mammary epithelium in order for alveolar development to proceed normally.**

The mouse provides a useful model to study mammary gland development. At the onset of puberty, a simple system of branching ducts begins growing out from the nipple area into a pad of fatty connective tissue that underlies the skin. During the luteal phase of the estrous cycles, the ductal system becomes more complex through the growth of side branches. Ductal side-branching becomes more extensive during early pregnancy, and subsequently alveolar bodies develop from these ducts, fill up the fat pad, and differentiate to become the sites of milk production.

The serum levels of the sex steroid progesterone are elevated during diestrus, the phase of luteal activity of the estrous cycle, and pregnancy. Moreover, experimental manipulation of the hormonal system has implicated this hormone as an essential stimulus required for the induction of ductal branching and for alveologenesis (1). However, the elucidation of the role of progesterone is complicated by the fact that, in the mammary epithelium, synthesis of the progesterone receptor (PR) depends on estrogen, the serum levels of which are also elevated during puberty and pregnancy. This has made it difficult to assess which developmental effects can be attributed to progesterone alone.

To dissect the role of progesterone from that played by estrogen, we generated mice lacking the PR by targeted inactivation of the PR gene in the mouse germ line (2). The mammary glands of the resulting young virgin $PR^{-/-}$ females show the same extent of ductal development as is seen in wild-type (wt) female mice (2). However, when wt and $PR^{-/-}$ virgin females were exposed to estradiol and progesterone, the wt breast tissue responded with side-branching and lobuloalveolar development, whereas the mammary glands of $PR^{-/-}$ females remained essentially unchanged. This suggested that PR is not required for initial ductal growth but is essential for subsequent side-branching and alveologenesis.

The administration of exogenous estrogen and progesterone, as was done in the above-described experiments and in a subsequent study extending this work (3), did not permit us to properly gauge the full spectrum of complex hormonal changes that occur during a normal pregnancy. During this period, the serum levels of a wide array of other hormones, including growth hormone, prolactin, placental lactogen, and adrenal steroids, are elevated. Moreover, the secretion of each of these hormones follows specific diurnal rhythms, and it is unlikely that injections of exogenous hormones achieve physiologic serum levels and correct local concentrations.

For these reasons, we resorted to transplanting $PR^{-/-}$ mammary tissues into wt animals that were subsequently impregnated. This allowed us to study the morphogenesis of the breast tissue in a hormonal environment that faithfully recapitulated that seen in pregnant, unmanipulated, wt animals. The results of previous research did not provide us with clear predictions of the outcomes of these transplantation experiments. For example, the PR is expressed in both stromal and epithelial compartments of the mammary gland (4). Within the epithelium, the distribution of the PR is variegated (5). Together, such observations provided no clear indication of the contributions of various subtypes of stromal and epithelial cells to mammary epithelial morphogenesis occurring in the presence or absence of the PR.

By grafting $PR^{-/-}$ epithelium or stroma in combination with PR wt stroma or epithelium, we have found that the primary target for progesterone is the mammary epithelium, while a direct response of the mammary stroma is not required in order for side-branching and lobuloalveolar development to occur. Furthermore, $PR^{-/-}$ mammary epithelial cells can give rise to alveoli when placed in close vicinity to PR wt epithelial cells, indicating that progesterone does not need to act directly on the alveolar cells and instead can orchestrate the morphogenetic and proliferative events of alveologenesis by affecting nearby cells in the mammary epithelium.

MATERIALS AND METHODS

Mice. ROSA26 and $RAG1^{-/-}$ mice were purchased from The Jackson Laboratory. The PR mutant mice were described

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Abbreviations: PR, progesterone receptor; wt, wild-type; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; MEC, mammary epithelial cell.

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elsewhere (2); transcription of both A and B forms of the PR was disrupted. All mice were bred in 129SV/C57BL6 genetic background.

For PR genotyping, genomic DNA was isolated from tails and analyzed by PCR. PCR was performed by denaturing the DNA at 94°C for 1 min, followed by 30 cycles of amplification: 94°C for 1 min, 60°C for 2 min, 72°C for 1 min, and a final extension step at 72°C for 5 min. The following PR-specific primers were used: *P1* (5′-TAG ACA GTG TCT TAG ACT CGT TGT TG-3′), *P2* (5′-AGC AGA AAA CCG TGA ATC TTC-3′), and a *neo* gene-specific primer, *N2* (5′-GCA TGC TCC AGA CTG CCT TGG GAA A-3′).

Presence of the β-galactosidase transgene was tested for by subjecting a piece of tail to the 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining procedure described below.

Whole-Breast Transplant. Four- to 6-week-old $PR^{+/+}$ or $PR^{-/-}$ female mice were sacrificed and their inguinal mammary glands were dissected. $RAG1^{-/-}$ females of the same age were anesthetized with Avertin i.p. (6). The ventral skin was incised and the abdominal muscle wall was exposed. A $PR^{-/-}$ and a $PR^{+/+}$ mammary gland were placed onto the abdominal wall and the incision was closed with surgical staples. Three weeks after surgery the recipients were mated. They were sacrificed at parturition. The two transplanted glands and an endogenous mammary gland were analyzed by whole-mount microscopy.

Fat-Pad Transplant. Three-week-old $PR^{+/+}$, $PR^{+/-}$, and $PR^{-/-}$ females were sacrificed and their inguinal mammary glands were exposed. The nipple-near region was removed. Into the remaining empty fat pad we injected primary mammary epithelial cells derived from ROSA26 females. The engrafted fat pads were placed onto the abdominal muscle wall of virgin $RAG1^{-/-}$ females.

Transplantation of Mammary Epithelium. The fat pads of 3-week-old $RAG1^{-/-}$ females were cleared (see above). Pieces of mammary tissue of 1-mm diameter were removed from the nipple region of $PR^{+/+}$ and $PR^{-/-}$ females and implanted as described before (7). Alternatively, the cleared fat pads were injected with $PR^{+/+}$ and $PR^{-/-}$ primary cells, cultured as described in ref. 8.

Mammary Gland Whole Mounts. The inguinal mammary glands were dissected, spread onto a glass slide, fixed in a 1:3 mixture of glacial acetic acid/100% ethanol, hydrated, stained overnight in 0.2% carmine (Sigma) and 0.5% $AlK(SO_4)_2$, dehydrated in graded solutions of ethanol, and cleared in 1:2 benzyl alcohol/benzyl benzoate (Sigma) as described previously (9).

Pictures were taken on a Leica MZ12 stereoscope with Kodak Ektachrome 160T.

X-Gal Staining. The transplanted mammary glands were dissected, fixed for 1 hr in 4% formaldehyde in phosphate-buffered saline (PBS), washed three times over 3 hr with rinse buffer (2 mM $MgCl_2$/0.1% sodium deoxycholate/0.2% Nonidet P-40 in PBS) and rotated in X-Gal staining solution (1 mg/ml X-Gal, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in rinse buffer) at 37°C for 18 hr, washed in PBS, and processed for whole-mounting as described above.

Histological Examination and Immunohistochemistry. For histological examination of the alveolar structures the whole-mounted mammary glands were washed in 100% ethanol prior to paraffin embedment. Sections were cut at 10 μm. Anti-β-casein antiserum (10) was diluted 1:500 and applied overnight at 4°C. Biotinylated secondary antibodies were detected with a Vectastain ABC kit (Vector Laboratories).

RESULTS

Development of the Mammary Gland During Pregnancy in the Absence of the PR. To analyze the role progesterone plays in the mammary gland during normal pregnancy, entire mammary glands from $PR^{-/-}$ female mice and their wt littermates were transplanted onto the abdominal muscle wall of PR wt females. The transplanted glands included both epithelial and stromal compartments. The recipient females were of the same 129SV/C57BL6 genetic background and were homozygous for the inactivated RAG1 allele (11). Females of this genotype are immunocompromised and therefore able to accept allografts. The engrafted females were mated 3 weeks after surgery and sacrificed immediately after a completed pregnancy. In all cases, the implants along with an endogenous mammary gland were analyzed by whole-mount microscopy.

While the wt implants and endogenous glands (Fig. 1 *Center* and *Right,* respectively) showed full alveolar development at parturition, the $PR^{-/-}$ grafts developed only a simple ductal system (Fig. 1 *Left*). These observations validated the transplantation procedure. More significantly, they demonstrated, as suggested by previous reports (1, 12), that progesterone is essential for side-branching and lobuloalveolar growth and showed that, in the absence of the PR, the mammary gland fails to undergo substantial proliferation in the presence of the full array of pregnancy-associated hormones.

Involvement of the Stromal and the Epithelial Compartments in PR-Mediated Responses. To address the question of whether progesterone acts on the mammary stroma or epithelium, engrafted animals were created in which either the mammary epithelium or the fat pad lacked PR because of inactivation of the PR gene. The development of the mammary gland in response to physiological hormonal stimulation was then followed.

In the mouse, the mammary epithelium grows out from the nipple into a fat pad that underlies the skin. At three weeks after birth, the epithelium of the gland has not yet penetrated extensively into the stroma and can be eliminated by removing the nipple region of the mammary gland (7). Mammary epithelial cells (MECs) that are introduced into the remaining "cleared" fat pad will give rise to a new ductal system. They can grow out from a piece of breast tissue that is placed into the fat pad (7, 13), or from single-cell suspensions that are injected into the fat pad (14).

We adapted these surgical procedures to create mammary glands that specifically lacked the PR in their stromal cells. Briefly, the nipple regions containing the mammary epithelium were removed from the fourth mammary glands of 3-week-old $PR^{-/-}$ females and their wt littermates. The resulting cleared fat pads were then implanted with mammary epithelium derived from a wt donor. Subsequently, the resulting reconstituted mammary glands were dissected and transplanted onto the abdominal muscle wall of $RAG1^{-/-}$ females.

We validated this transplantation procedure by implanting PR wt epithelium into PR wt fat pads. The resulting engrafted glands developed like the endogenous mammary glands in virgin as well as postpartum recipients, demonstrating that the

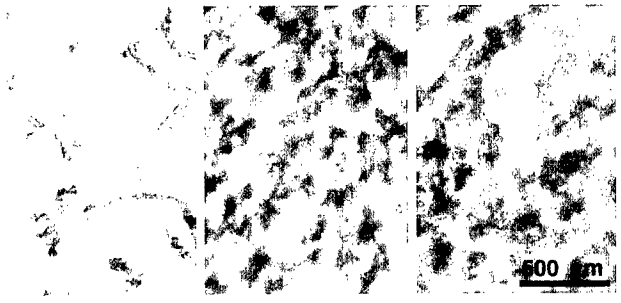

FIG. 1. Whole breast transplantation. Whole-mount preparations of the $PR^{-/-}$ (*Left*) and $PR^{+/+}$ (*Center*) whole breast implant and endogenous mammary gland (*Right*) derived from $RAG1^{-/-}$ recipient mouse after parturition.

engrafted fat pad had become fully vascularized when transplanted in this fashion.

The interpretation of these experiments depended upon our ability to distinguish implanted mammary epithelium from any residual endogenous epithelium that inadvertently had not been removed during the preparation of the cleared mammary fat pads. In fact, in the virgin gland, it is easy to distinguish ducts arising from implanted epithelium from those that are endogenous to this gland because of the distinctive orientations of ductal growth. Thus, the endogenous epithelium grows unidirectionally from the nipple into the fat pad, whereas the ducts arising from the implant, which we place into the center of the cleared fat pad, grow centrifugally. At parturition, however, when the fat pad is filled with alveoli, it is difficult to distinguish the two ductal systems, making it impossible to rule out that the observed epithelial structures derive from residual endogenous epithelium.

To address this difficulty, mammary epithelium derived from ROSA26 female mice was exploited (15). Mice of this transgenic strain express the β-galactosidase gene in virtually all their tissues. The mammary epithelium of these ROSA26 mice was implanted into the cleared fat pads of wt mice. When these reconstituted fat pads were subjected to an X-Gal staining procedure, the implanted ROSA26-derived epithelium turned blue and could thus be unequivocally distinguished from any endogenous epithelium, which was visualized by the red color of the carmine/alum counterstain. Together, the above-described preliminary experiments and the use of ROSA26 cells validated our transplantation procedures and our ability to study engrafted tissues without the confounding effects of residual tissue originating from the recipient breast.

The above procedures were utilized to resolve the respective roles of stroma- and epithelium-derived PR populations in mammary gland proliferation and differentiation. First, ROSA26.$PR^{+/+}$ epithelium was transplanted into cleared $PR^{-/-}$ fat pads; the resulting reconstituted mammary glands were then placed onto the abdominal muscle wall of a $RAG1^{-/-}$ recipient female. Four weeks later, the engrafted $RAG1^{-/-}$ recipients were mated. After they had given birth, the transplanted mammary gland and an endogenous mammary gland were analyzed by whole-mount microscopy. As can be seen in Fig. 2, the injected ROSA26-derived mammary epithelial cells grew equally well in transplanted fat pads from wt (Fig. 2 *Right*) and $PR^{-/-}$ (Fig. 2 *Left*) donors. This result demonstrated that the presence of the PR in the mammary stroma was not essential for the pregnancy-induced side-branching and lobuloalveolar development.

Next, we assessed the role of the PR in the epithelium independent of its function in the stroma. To do this, mammary epithelial cells derived from either $PR^{-/-}$ or wt donors were transplanted into the cleared mammary fat pads of wt recipients. The engrafted recipients were mated and their mammary glands were analyzed at parturition. The results of these experiments are shown in Fig. 3. Whereas the wt implant gave rise to a fully developed mammary tree, the epithelium lacking the PR grew into only a simple ductal tree (Fig. 3 *Left*). Similarly, when we analyzed the mammary glands of engrafted virgin females 2 months after surgery, the wt implant as well as the endogenous breasts showed side-branching, whereas the $PR^{-/-}$ breast had only a simple ductal system (Fig. 3 *Right*). Table 1 summarizes the results of these transplantation experiments. These results allowed us to conclude that the mammary epithelium is the prime target of progesterone both before and during pregnancy, and that a direct response of the mammary stroma to progesterone does not play an essential role.

Role of the PR in the Development of Alveoli. The experiments above indicated that the absence of the PR from all cells of the mammary epithelium resulted in a failure of side-branching and lobuloalveolar growth. However, they did not address the question of whether the presence of PR was required in all cells of the ductal epithelium or in only a subset of MECs in order for these morphogenetic processes to proceed normally.

To distinguish between these possibilities, we created mosaic mammary epithelia containing both $PR^{-/-}$ and $PR^{+/+}$ MECs. The latter cells were derived from ROSA26 mice. In this case, tissue structures composed of $PR^{+/+}$ cells would turn blue upon X-Gal staining when analyzed by whole-mount microscopy. Structures composed of $PR^{-/-}$ cells would turn red, being stained only by the carmine/alum counterstain.

Mixtures of $PR^{+/+}$ and $PR^{-/-}$ MECs in different ratios were injected into the cleared mammary fat pads of $RAG1^{-/-}$ females. These mixtures were obtained either by combining single-cell suspensions derived from $PR^{-/-}$ and $PR^{+/+}$.ROSA26 primary cultures or by mixing finely minced mammary tissues dissected from females of these two strains. Two months later, the engrafted recipients were mated, and the engrafted breasts were analyzed toward the end of pregnancy.

Depending on the degree of homogeneity of the injected mixture and the ratio in which the cells of the different genotypes were mixed, we found two types of chimerism. In the

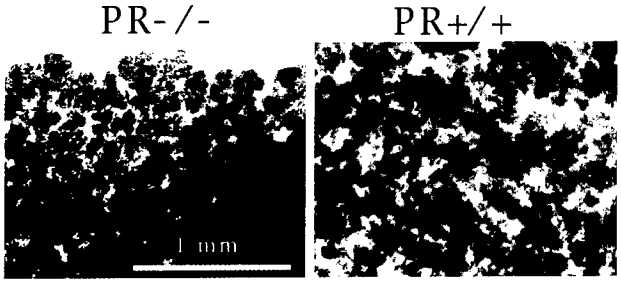

FIG. 2. Transplantation of engrafted fat pads. Whole-mount preparations of transplanted reconstituted breasts. Fat pads from $PR^{-/-}$ or $PR^{+/+}$ mice were engrafted with ROSA26 (β-galactosidase$^+$) $PR^{+/+}$ primary mammary epithelial cells and transplanted onto the abdominal muscle wall of $PR^{+/+}$.$RAG1^{-/-}$ recipients. the reconstituted mammary glands were removed from the recipients after parturition and stained with X-Gal before whole-mounting.

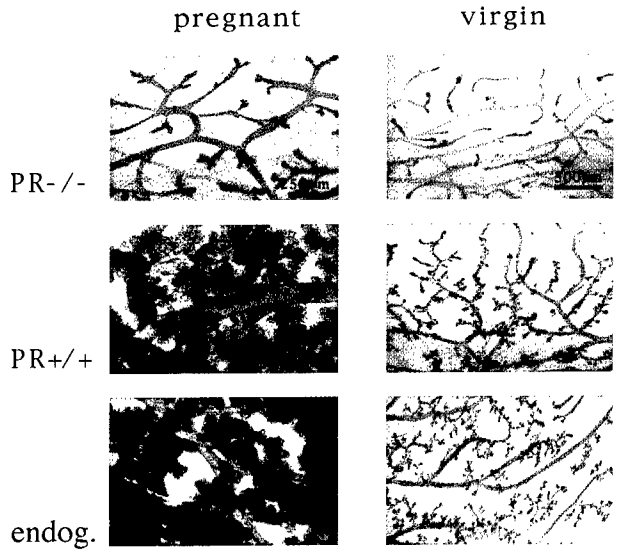

FIG. 3. Transplantation of epithelium. Whole-mount preparations of mammary glands from $PR^{+/+}$.$RAG1^{-/-}$ recipients. (*Left*) Preparation derived from a recipient after parturition. (*Right*) Preparation derived from a virgin mouse. (*Top*) Transplanted $PR^{-/-}$ epithelium. (*Middle*) Transplanted $PR^{+/+}$ epithelium. (*Bottom*) Endogenous mammary gland.

Table 1. Requirement of the PR in the stroma and/or the epithelium for alveolar development in mammary transplants analyzed post partum

Transplant	No. samples with alveolar growth/no. successful transplants
Mammary glands *in toto*	
Stroma $PR^{+/+}$/epithelium $PR^{+/+}$	8/8
Stroma $PR^{-/-}$/epithelium $PR^{-/-}$	0/8
Fat pad injected with $PR^{+/+}$ROSA26 epithelium cells	
Stroma $PR^{+/+}$/injected epithelium $PR^{+/+}$	6/6
Stroma $PR^{+/-}$/injected epithelium $PR^{+/+}$	8/8
Stroma $PR^{-/-}$/injected epithelium $PR^{+/+}$	6/6
Epithelium	
Stroma (host) $PR^{+/+}$/epithelial transplant $PR^{+/+}$	13/13
Stroma (host) $PR^{+/+}$/epithelial transplant $PR^{-/-}$	0/13

first type, the mammary glands showed discrete sectors having distinct phenotypes. An example, representative of 17 samples of this type of chimerism, is shown in Fig. 4. One half of the epithelial component of the mammary gland stained red while the other half stained blue; this indicated the origins of these two sectors from $PR^{-/-}$ and ROSA26 engrafted cells respectively. The sector composed of the $PR^{-/-}$ cells represents a simple ductal tree, whereas the sector composed of the $PR^{+/+}$.ROSA26 cells shows extensive lobuloalveolar growth. This result demonstrated that the coexistence of MECs of $PR^{+/+}$ and $PR^{-/-}$ in one fat pad is not sufficient to rescue the morphogenetic defect intrinsic to the $PR^{-/-}$ cells.

Most of the chimeric epithelia that arose from single-cell suspensions in which the wt cells were in 10-fold excess over $PR^{-/-}$ cells showed complete lobuloalveolar development. However, at higher magnification distinct red alveoli and blue alveoli could be identified. This observation suggested but did not prove that $PR^{-/-}$ cells could participate in alveolar formation if they were in close proximity with wt MECs.

Any conclusions concerning the ability of the $PR^{-/-}$ MECs to form alveoli were clouded by the possibility that certain $PR^{+/+}$.ROSA26 cells that participated in alveologenesis had failed to stain blue, thereby taking on the appearance of the $PR^{-/-}$ cells in the same mixed grafts. To address this issue, we crossed the β-galactosidase transgene into the $PR^{-/-}$ genetic background. By transplanting $PR^{-/-}$.ROSA26 mammary epithelium into wt recipients and analyzing the transplanted glands after birth we were assured that the transgene did not affect the $PR^{-/-}$ phenotype (data not shown). Subsequently, suspensions of $PR^{-/-}$.ROSA26 MECs were mixed with $PR^{+/+}$ MECs lacking the β-galactosidase transgene to generate chimeric breasts. On this occasion, we looked for a result opposite to that seen previously—alveolar cells that stained blue. Indeed, as shown in Fig. 4 *Center*, a representative of 26 independent grafts, the mammary glands obtained from pregnant engrafted females showed areas with blue alveoli, proving conclusively that $PR^{-/-}$ cells can participate in the formation of alveoli if they are in close vicinity to wt epithelial cells.

To determine whether the alveolar structures constituted by $PR^{-/-}$ cells are functional we assessed their morphology on histological sections. As shown in Fig. 4 *Right*, the lumina of the blue $PR^{-/-}$ alveoli compare with those of wt alveoli, indicating the presence of secreted material. Similarly, secretory vacuoles are present. Immunostaining with anti-β-casein antibody revealed the expression of the milk protein (arrow, Fig. 4 *Upper Right*). Together these results indicate that the $PR^{-/-}$ alveoli are fully differentiated. Thus, the presence of the PR is required in only a portion of the MECs in order for lobuloalveolar development to occur. Moreover, these findings suggest that progesterone activates a paracrine signaling route that operates between distinct subtypes of MECs, permitting $PR^{-/-}$ MECs to participate directly in lobuloalveolar proliferation and differentiation.

DISCUSSION

Hormonal ablation/reconstitution experiments (1) have suggested that progesterone plays an important role in the changes that the mammary gland undergoes during early pregnancy,

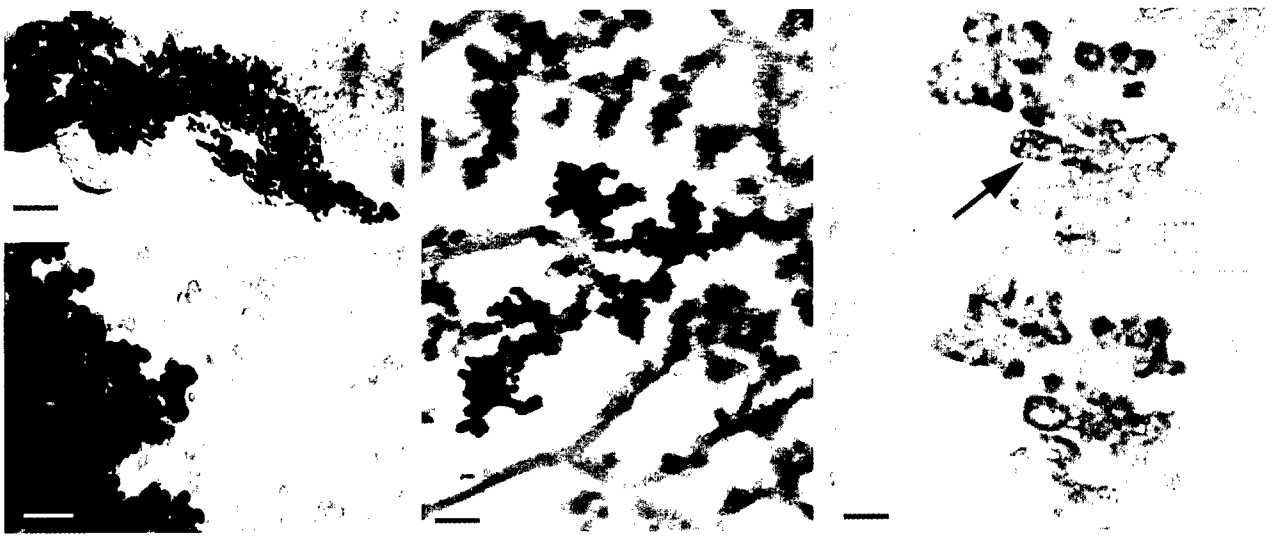

FIG. 4. Rescue of the $PR^{-/-}$ phenotype in $PR^{-/-}$ and $PR^{+/+}$ chimeric epithelia. (*Left*) Whole-mount preparation of cleared $PR^{+/+}$.$RAG1^{-/-}$ fat pad implanted with a mixture of $PR^{-/-}$ (red) epithelium and ROSA26.$PR^{+/+}$ epithelium (blue) in a 1:1 ratio. The engrafted mammary gland was removed after the recipient had given birth, subjected to X-Gal staining, and whole-mounted.(Bar in *Upper* corresponds to 2 mm; bar in *Lower*, to 200 μm). (*Center*) Whole-mount preparation of cleared $PR^{+/+}$.$RAG1^{-/-}$ fat pad injected with a mixture of $PR^{-/-}$.ROSA26 (blue) epithelium and $PR^{+/+}$ epithelium (red) injected in a 1:10 ratio, treated as for *Left*. (Bar corresponds to 200 μm.) (*Right*) Adjacent histological sections of an area with $PR^{-/-}$.ROSA26 alveolar structures. (*Upper*) Expression of β-casein in wt and $PR^{-/-}$.ROSA26 alveoli. (*Lower*) Control without primary antibody. Arrow indicates $PR^{-/-}$.ROSA26 alveolus expressing β-casein. (Bar corresponds to 50 μm.)

namely side-branching and initial alveolar growth. To determine the extent to which progesterone signaling is limiting in development, we generated mice lacking the PR gene (2). However, because the $PR^{-/-}$ females have multiple impairments in their reproductive functions, the specific consequences of PR inactivation on mammary gland development could not be assessed in these mice.

To circumvent this difficulty, we have used various transplantation techniques to elucidate the role of progesterone in the development of the mammary gland. In particular, we have made use of cells derived from mice carrying the β-galactosidase transgene. These cells turn blue upon X-Gal staining, making it possible to distinguish these cells histochemically from neighboring β-galactosidase-negative cells. In one experiment, this allowed us to distinguish the β-galactosidase-positive implanted MECs from the β-galactosidase-negative endogenous cells of an engrafted breast; in another setting, this procedure made it possible for us to distinguish MECs carrying two functional PR alleles from those lacking the PR.

Most transplantation experiments involving nonsyngeneic grafts have exploited nude mice as recipients. We note here in passing the utility of the $RAG1^{-/-}$ mice used for transplantation experiments designed to elucidate mammary gland physiology. Because nude mice have low estrogen levels, they do not represent good recipients in transplantation experiments designed specifically to gauge mammary function. In contrast, the $RAG1^{-/-}$ mice used here exhibit developmental defects that are strictly limited to B and T cell development (11).

Our initial experiments involving the transplantation of $PR^{-/-}$ mammary glands into $PR^{+/+}.RAG1^{-/-}$ females were motivated by the need to assess the role of the PR in an *in vivo* physiologic environment in which the full array of pregnancy-associated hormonal signals was present. $PR^{-/-}$ mammary glands grafted into a $PR^{+/+}.RAG1^{-/-}$ recipient developed only a simple ductal system, even when the host went through a series of estrous cycles and a normal pregnancy. This indicated that side-branching and lobuloalveolar growth rely on the presence of the PR, and that other signaling mechanisms operating in the breast tissue cannot compensate for the absence of the PR to allow these processes to proceed normally.

These initial results left us with two distinct scenarios. In one, both side-branching and lobuloalveolar proliferation, each in its own right, depends on the presence of progesterone. In the other, side-branching depends on progesterone, whereas lobuloalveolar growth depends on prior side-branching and is therefore only indirectly dependent on progesterone. Our analysis of a series of whole mounts of mammary glands from wt pregnant mice showed that alveoli sprouted not only from side branches (secondary ducts) but also from the primary ducts (data not shown). This finding indicated that side-branching is not an absolute prerequisite for alveolar growth. For this reason, we concluded that the PR is required for lobuloalveolar proliferation *per se* in addition to its demonstrated role in side-branching.

We next addressed the issue of whether progesterone needs to act on the mammary stroma, the epithelium, or both. One important clue for resolving this puzzle appeared to come from the longstanding observation that morphogenesis in many epithelial-mesenchymal organs such as the mammary gland is controlled by inductive events (16) that require cross-talk between epithelial and stromal components. In the breast in particular, the embryonic mammary mesenchyme induces the overlying epithelium to develop into the mammary bud (17). Moreover, in male embryos of various mouse strains, androgens act on the stroma to induce the involution of the mammary anlage (18, 19). The estrogen receptor is required in the mammary stroma for ductal growth to occur (20).

The role of the stroma in mediating progesterone-dependent processes in the breast has been less clear. For example, ligand-binding studies have shown that 80% of the progesterone receptors in the mouse mammary gland localize to the epithelium, while the remaining 20% are found in the stroma (4). Such observations have been compatible with models in which the epithelial cells, the stromal cells, or both cell types are required to mediate the direct responses to progesterone.

More recently, epithelial/stromal reciprocal transplantations between wt and estrogen receptor $(ER)^{-/-}$ and wt and $PR^{-/-}$ tissues have demonstrated that stromal derived ER and PR exert paracrine effects on the epithelium both in the uterus (21) and in the vagina (G. R. Cunha and B.W.O., unpublished observations). We show here that mammary glands lacking PR in the stroma undergo normal development, whereas the absence of the PR from the epithelium confers the $PR^{-/-}$ phenotype, indicating that the target cells of progesterone in the mammary gland are in the epithelium. While effects of progesterone on the mammary stroma cannot be excluded, they do not appear to contribute in any obvious way to the development of the ductal tree and alveoli.

Recently reported experiments in which we participated (3) yielded results that are in conflict with one aspect of the present work. These previous experiments appeared to indicate that the PR that functions within the stromal compartment exerts an effect on epithelial ductal growth, contrary to the present results, which indicate the opposite. We find the present results more compelling for several reasons. The number of transplanted animals examined here was much larger. Moreover, we have analyzed the behavior of mammary glands in a situation in which the only PR-negative tissue in engrafted animals was the mammary stroma; the earlier work, in contrast, examined the behavior of wt epithelium transplanted into the cleared $PR^{-/-}$ fat pad of a $PR^{-/-}$ host. In concordance with our conclusion, a recent immunostaining failed to detect any PR protein in the fat pad (22).

The present work together with previous observations of others (1, 12) indicates that progesterone is required for two distinct morphogenetic processes in the breast—side-branching and preparation of ductal cells for subsequent lobuloalveolar development. The precise mechanisms by which progesterone enables ductal MECs to participate in alveologenesis has been unclear. The pattern of PR expression in the mammary epithelium is inhomogeneous (5), suggesting the involvement of only a subset of ductal cells in progesterone-triggered processes. The connected issue of whether the PR-expressing cells represent the precursors of the alveolar outgrowths is addressed here.

Our observation that $PR^{-/-}$ cells can give rise to alveolar structures if they are in close vicinity to $PR^{+/+}$ cells indicates that progesterone does not need to act directly on a ductal epithelial cell for it to participate in alveolar formation. Instead, it appears that progesterone acts on a subtype of ductal cell, causing it to release paracrine signals that permit other nearby epithelial cells to participate directly in lobuloalveolar proliferation.

The present work provides no indication about the nature of the paracrine signal released by the progesterone-activated ductal cell. However, the observation that close apposition of PR-positive with PR-negative cells is required to rescue the $PR^{-/-}$ phenotype indicates that the signal, whatever its biochemical nature, is transmitted only over short intercellular distances. Factors that are tightly associated with the extracellular matrix such as wnt proteins and fibroblast growth factors, which are differentially expressed during mammary gland development (23, 24), are attractive candidates for conveying such paracrine signals.

Our data provide no indication whether or not these paracrine signals communicate directly between the progesterone-

activated ductal cells and closely apposed alveolar precursor cells. It remains equally possible that the progesterone-activated ductal cell communicates with the stroma; the latter, in turn, may pass on a signal directly to the alveolar precursor cells as suggested by others (25). The use of tissue reconstitution techniques and genetically altered cells should allow the further dissection of the molecular mechanisms of mammary morphogenesis over the next several years.

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RESEARCH COMMUNICATION

Essential function of *Wnt-4* in mammary gland development downstream of progesterone signaling

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Female reproductive hormones control mammary gland morphogenesis. In the absence of the progesterone receptor (PR) from the mammary epithelium, ductal side-branching fails to occur. We can overcome this defect by ectopic expression of the protooncogene *Wnt-1*. Transplantation of mammary epithelia from *Wnt-4*$^{-/-}$ mice shows that Wnt-4 has an essential role in side-branching early in pregnancy. *PR* and *Wnt-4* mRNAs colocalize to the luminal compartment of the ductal epithelium. Progesterone induces *Wnt-4* in mammary epithelial cells and is required for increased *Wnt-4* expression during pregnancy. Thus, Wnt signaling is essential in mediating progesterone function during mammary gland morphogenesis.

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Development of the mammary gland occurs largely postnatally under the control of the female reproductive hormones estrogen, progesterone, and prolactin (Nandi 1958). A system of ducts grows outward from the nipple into the mammary fat pad that lies under the skin. The ducts then elongate and bifurcate during puberty until they reach the edges of the fat pad (Daniel and Silberstein 1987). Subsequently, with recurrent estrous cycles and during early pregnancy the ductal system increases in complexity through the addition of sidebranches that sprout from the preexisting ducts (Daniel and Silberstein 1987). The mechanisms that enable the systemic factors to control locally acting factors involved in these morphogenetic events remain largely unknown. Recently, we and others have shown that progesterone acts via the progesterone receptor (PR) in the mammary epithelium to induce side-branching (Lydon et al. 1995; Humphreys et al. 1997; Brisken et al. 1998) and that it does so by a paracrine mechanism (Brisken et al. 1998).

We speculated that Wnt proteins might function as the paracrine factors that operate downstream of progesterone and the PR to mediate the process of side-branching. Wnt proteins have important roles in the development of various vertebrate and invertebrate tissues (Nusse and Varmus 1992; Cadigan and Nusse 1997). These factors are secreted glycoproteins that bind to members of the Frizzled family of seven-transmembrane-domain receptors. Several *Wnt* genes can function as oncogenes in the mouse breast when their transcription is activated by insertion of the provirus mouse mammary tumor virus (MMTV) (Nusse and Varmus 1982; Roelink et al. 1990; Lee et al. 1995) or when they are expressed ectopically (Tsukamoto et al. 1988).

Results and Discussion

To test whether a Wnt factor might function downstream of progesterone signaling in triggering ductal side-branching in the breast, we crossed mice carrying an MMTV LTR-driven *Wnt-1* transgene (Tsukamoto et al. 1988) with mice heterozygous for a previously described inactivating mutation at the *PR* locus (Lydon et al. 1995), to generate *Wnt-1* transgenic females that were either $PR^{-/-}$or $PR^{+/+}$. We then sought to test whether the ectopically expressed Wnt-1 protein might restore the side-branching that is lacking in $PR^{-/-}$ mammary ducts (Fig. 1A).

Mammary epithelia were removed from mice of both genotypes and transplanted into the inguinal fat pads of 3-week-old $PR^{+/+}$ females. These fat pads previously had been surgically cleared of endogenous epithelium. When epithelial tissue (DeOme et al. 1959) or primary cells (Daniel and DeOme 1965) are engrafted into such cleared fat pads, they are able to form a new ductal system. These recipient females were also mutant at the RAG1 locus ($RAG1^{-/-}$), as these mice are immunocompromised and therefore able to accept allografts (Mombaerts et al. 1992; Brisken et al. 1998).

Ten weeks after grafting, control unmanipulated mammary glands in these recipient females showed a simple ductal system characteristic of a 13-week-old virgin mouse. However, the fat pads carrying implanted $PR^{+/+}$. MMTV *Wnt-1*^{tg} and $PR^{-/-}$ MMTV *Wnt-1*^{tg} epithelia showed increased side-branching (Fig. 1B). Thus, ectopic expression of *Wnt-1* can induce side-branching in a $PR^{-/-}$ epithelium in which side-branching is defective, suggesting that Wnt signaling can mimic this progesterone-induced response and may therefore act downstream of the PR.

We reported previously that in chimeric epithelia derived from mixed wild-type and $PR^{-/-}$ mammary epithelial cells (MECs), the branching defect of the mutant MECs could be rescued if these cells grew in close proximity to their wild-type counterparts (Brisken et al. 1998). This suggests that progesterone elicits its morpho-

[*Key Words*: Mammary gland; morphogenesis; Wnt-4 signaling; side-branching, progesterone function]
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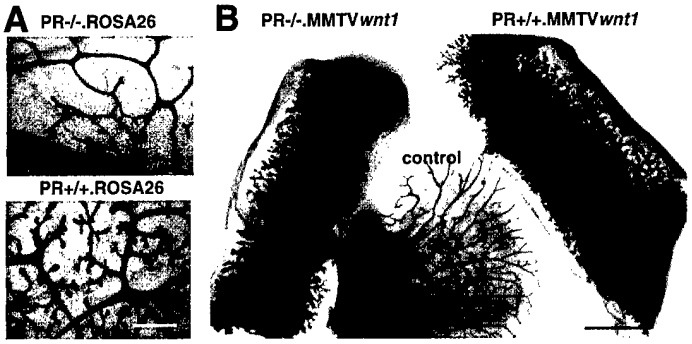

Figure 1. Side-branching in the presence of *Wnt-1* and absence of the PR. (*A*) Intrinsic side-branching defect in $PR^{-/-}$ mammary epithelium. Mammary epithelium was harvested from $PR^{-/-}$ *ROSA26* (*top*) and $PR^{+/+}$ *ROSA26* (*bottom*) 10-week-old female mice and engrafted to the cleared fat pads of 3-week-old F_1 (129SV/C57B16) recipients. Shown are whole-mount preparations of mammary glands subjected to X-gal stain, from a recipient at day 12 of pregnancy (10 weeks after surgery). Results were similar to those reported previously. Bar, 400, μm. (*B*) Constitutive side-branching of MMTV *Wnt-1*^{tg} irrespective of the PR status. Mammary epithelium was harvested from $PR^{-/-}$ MMTV *Wnt-1*^{tg}and $PR^{+/+}$ *MMTV Wnt-1*^{tb} 10-week-old female mice and engrafted to the cleared fat pads of 3-week-old recipients. Shown are whole-mount preparations of mammary gland from a virgin $RAG1^{-/-}$ recipient 10 weeks after surgery. (*Left*) Inguinal fat pad engrafted with $PR^{-/-}$ MMTV *Wnt-1*^{tg} mammary epithelium; (*right*) inguinal fat pad engrafted with $PR^{+/+}$ MMTV *Wnt-1*^{tg} mammary epithelium; (*center*) thoracic mammary gland, as an ungrafted endogenous control. Identical results were obtained in 16 independent grafts of $PR^{-/-}$ MMTV *Wnt-1*^{tg} and control mammary epithelium. Bar, 5 mm.

genetic effects, at least in part, by causing PR-positive MECs to release a factor that acts over short distances on other cells within the breast.

To test whether Wnt-1 also acts in a paracrine fashion to induce side-branching, we mixed MMTV *Wnt-1*^{tg} MECs with MECs derived from *ROSA26* mice (Friedrich and Soriano 1993). These latter cells carry a *lacZ* transgene, which makes their identification possible upon whole mount analysis of breast tissue. As expected, the MMTV *Wnt-1*^{tg} cells, stained in red, showed increased side-branching (Fig. 2). In addition, the blue wild-type cells carrying the *lacZ* transgene, located adjacent to these MMTV *Wnt-1*^{tg} MECs, also showed increased side-branching. This indicates that secreted Wnt-1 is sufficient to cause side-branching and that Wnt-1, like the factor released by PR-positive cells, acts in a paracrine fashion to induce side-branching. When wild-type MECs were mixed with MECs derived from *ROSA26* mice, ductal branching was not affected (data not shown), indicating that the increased branching is not induced by experimental manipulation.

Although these experiments indicate that a Wnt protein was sufficient for side-branching, being able to mimic the morphogenetic response normally elicited by progesterone, they did not resolve whether a Wnt factor has an essential role in the normal morphogenetic process. *Wnt-1* itself is not normally expressed in the mammary gland, but the related gene *Wnt-4* (Munsterberg et al. 1995; Kispert et al. 1998), which acts similarly to *Wnt-1* when ectopically expressed in the mammary epithelium (Bradbury et al. 1995), is expressed during the period when side-branching occurs in early to mid-pregnancy (Gavin and McMahon 1992; Weber-Hall et al. 1994). To evaluate the specific role of *Wnt-4* in mammary morphogenesis, we analyzed mammary epithelium from mice lacking both copies of the *Wnt-4* gene (Stark et al. 1994). These mice die perinatally due to kidney failure (Stark et al. 1994), precluding analysis of subsequent mammary development. Responding to this, we harvested the mammary buds from 14.5-day-old $Wnt\text{-}4^{-/-}$ and wild-type embryos and engrafted them into the cleared mammary fat pads of wild-type hosts. Both types of implants initially gave rise to normal ductal systems in virgin recipients (Fig. 3, left). However, at day 12 of pregnancy $Wnt\text{-}4^{-/-}$ implants showed substantially less ductal branching than their wild-type counterparts (Fig. 3, middle). Later in pregnancy, engrafted $Wnt\text{-}4^{-/-}$ epithelia began to resemble wild-type epithelial grafts, exhibiting a more normal pattern of arborization (Fig. 3, right). This may be explained by the actions of other members of the Wnt family of factors that are known to be expressed late in pregnancy, such as *Wnt-5a*, *Wnt-5b*, and *Wnt-6*.

In situ hybridization with *PR*- and *Wnt-4*-specific cRNA probes on sections of mammary glands from virgin mice and during early pregnancy (days 4 and 8) reveal that both molecules are expressed at low levels in the virgin and induced during pregnancy (Fig. 4, top). Higher magnifications illustrate that as reported

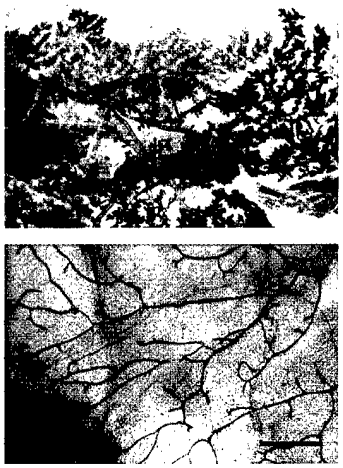

Figure 2. Paracrine induction of side-branching by Wnt-1. Primary mammary epithelial cells were derived from 10-week-old MMTV *Wnt-1*^{tg} and *ROSA26* females. After 5 days in vitro culture, the cells were trypsinized, mixed in a 1:1 ratio, and injected into cleared fat pads of 3-week-old $RAG1^{-/-}$ recipients. Mammary glands from $RAG1^{-/-}$ recipients at 10 weeks after surgery were subjected to X-gal stain, carmine alum counterstain, and mounted whole. (*Top*) Cleared fat pad reconstituted with a mixture of *ROSA26* (blue) and MMTV *Wnt1* (red) mammary epithelial cells; (*bottom*) ungrafted control, thoracic mammary gland. Bar, 500 μm. *Wnt-1* overexpressing cells (red) induce premature side-branching in wild-type cells (blue).

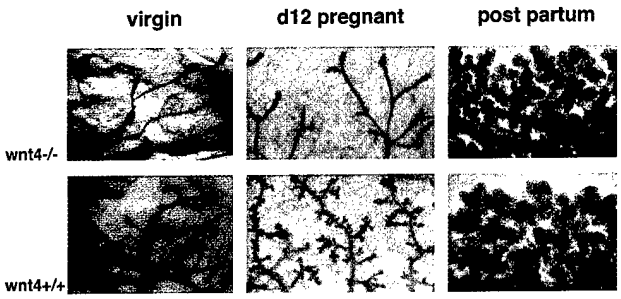

Figure 3. Function of *Wnt-4* in the mammary epithelium at mid-pregnancy. Mammary buds were prepared from *Wnt-4*$^{-/-}$ and wild-type littermates (129SV/C57B16 mixed genetic background) at E14.5 and engrafted to the cleared fat pads of 3-week-old F_1 (129SV/C57B16) recipients. At 10 weeks after surgery the mammary glands from virgin and impregnated recipients were analyzed by whole-mount preparation. (*Top*) Mutant grafts; (*bottom*) wild-type control grafts. Shown are mammary glands derived from a grafted virgin mouse (*left*), a mouse at day 12 of pregnancy (*center*), or at parturition (*right*). *Wnt-4*$^{-/-}$ epithelium fails to initiate side-branching at day 12 of pregnancy. Results were obtained with 4 virgin, 10 mid-pregnant, and 5 recipients at parturition. Bar 500 μm.

previously (Silberstein et al. 1996), the PR is not expressed in the myoepithelium but is restricted instead to the luminal epithelium (see arrows in Fig. 4, bottom) and that the same is true for *Wnt-4*. The same colocalization was observed on sections from the murine uterus during early pregnancy (data not shown). These observations of colocalized expression are consistent with a model that progesterone signaling induces *Wnt-4* expression.

To test whether *Wnt-4* expression is under the control of progesterone, we injected groups of ovariectomized mice with either 17-β-estradiol, 17-β-estradiol and progesterone, or the vehicle alone for 20 days as described (Said et al. 1997). 17-β-Estradiol injections were required to induce expression of the PR in MECs (Said et al. 1997). At the end of these treatments, one mammary gland from each mouse was analyzed by whole-mount microscopy to assess the morphology of the ductal system, enabling us to control for adequate gonadectomy in the vehicle-treated mice and to assess the efficacy of hormone replacement in the stimulated mice. RNA was extracted from a second mammary gland of each mouse and assayed by RT–PCR for levels of *GAPDH* and *Wnt-4* mRNA expression. We found a slight increase in the expression of *Wnt-4* mRNA in response to 17-β-estradiol treatment alone, but a three- to fivefold increase of *Wnt-4* mRNA following 17-β-estradiol and progesterone treatment (Fig. 5A).

The above results suggest that increased *Wnt-4* expression during pregnancy is under progesterone control. To test this possibility further, we assayed *Wnt-4* expression in the mammary glands of pregnant mice that had been engrafted with *PR*$^{-/-}$ epithelium in one fat pad and PR$^{+/+}$ epithelium in the contralateral fat pad. In both cases, the transplanted epithelial cells also carried a *lacZ* gene, enabling us to use RT–PCR analysis to gauge the level of RNA recovered from the engrafted epithelium of each reconstituted gland. At day 12 of pregnancy, a threefold difference between the levels of *Wnt-4* mRNA was consistently observed between the *PR*$^{-/-}$ implants and their wild-type counterparts (Fig. 5B), which compares to the induction of endogenous *Wnt-4* expression normally seen during pregnancy (Gavin and McMahon 1992; Weber-Hall et al. 1994; data not shown). The levels of *lacZ* mRNA were comparable between the two grafts. Thus, progesterone signaling is required within the grafted mammary epithelium for the induction of *Wnt-4* expression that is normally seen during pregnancy.

To test whether the induction of *Wnt-4* by progesterone is a direct effect of PR action on mammary epithelial cells, we treated primary MECs in culture with progesterone. As shown in Figure 5C, representative of eight independent experiments, *Wnt-4* RNA expression was significantly induced as early as 4–8 hr after progesterone

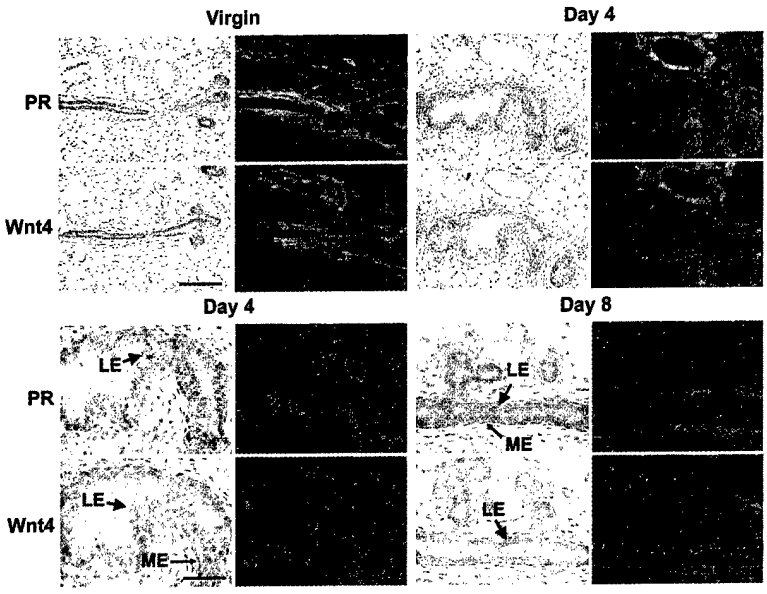

Figure 4. Coexpression of *PR* and *Wnt-4* mRNAs in the luminal mammary epithelium. Mammary glands were harvested from a virgin adult female mouse and from mice at days 4 and 8 of pregnancy. The glands were processed for in situ hybridization; adjacent sections were hybridized with ^{35}S-labeled antisense cRNA probes for *PR* or *Wnt-4* and exposed for 7 days. Hematoxylin- and eosin-stained sections are shown next to the corresponding dark-field exposures. (*Top*) Coordinated induction of *PR* and *Wnt-4* mRNAs during pregnancy. Low magnification (bar, 150 μm) of mammary gland showing increased signal intensity for both *PR* and *Wnt-4* mRNAs in the ductal epithelium at 4 days of pregnancy vs. virgin. (*Bottom*) Colocalization of *PR* and *Wnt-4* mRNA expression in the mammary luminal epithelium. High magnification (bar, 75 μm) of selections from mammary glands at days 4 and 8 of pregnancy showing that both *PR* and *Wnt-4* mRNA expression localizes to the luminal epithelium (LE) and is absent from the myoepithelium (ME). Light green areas represent the dense fibrous stroma surrounding the mammary ducts.

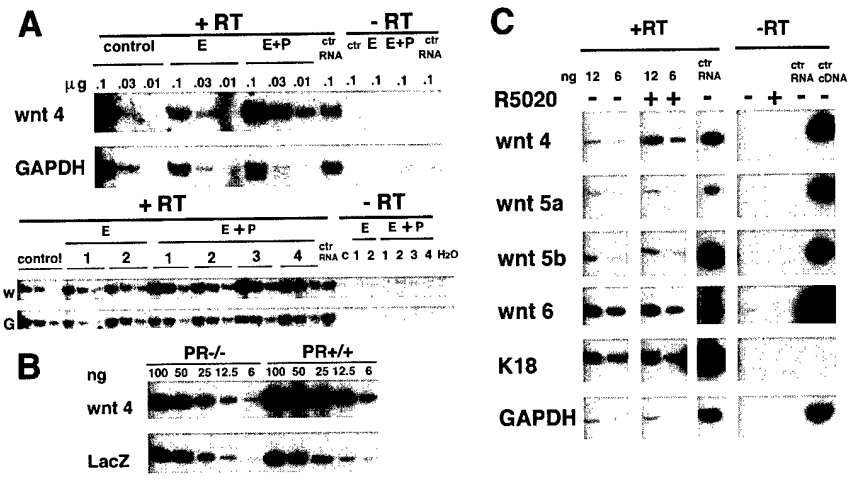

Figure 5. Induction of *Wnt-4* expression in the mammary epithelium in vivo and in vitro by progesterone. (*B*) Quantification of *Wnt-4* mRNA expression by semiquantitative PCR in mammary glands after 20 days of hormone treatment. Ten week-old virgin mice were ovarectomized. After 3 weeks they were injected for 20 days either with vehicle only (control), 10 µg of 17-β-estradiol (E)/day or 10 µg of estradiol and 100 µg of progesterone (E+P)/day. Total RNA was prepared from individual mammary glands, and samples in three serial dilutions, to ensure a linear signal response, were subjected to RT–PCR with primers specific for *Wnt-4* or *GAPDH*. The same amounts of RNA in three serial dilutions were analyzed in each case. The undiluted RNA subjected to PCR amplification yielded no signal. Shown are two independent experiments, one comprising three mice (*top*) and one comprising seven mice (*bottom*). The products were quantified by densitometric scanning. The ratio of *Wnt-4/GAPDH* of the progesterone-treated samples was three- to fivefold higher than the 17-β-estradiol-treated samples. (*B*) *Wnt-4* mRNA expression in mammary glands engrafted with $PR^{-/-}$ or $PR^{+/+}$ mammary epithelium. Mammary epithelium was harvested from $PR^{-/-}$ *ROSA26* and $PR^{+/+}$ *ROSA26* 10-week-old female mice and engrafted to the cleared fat pads of 3-week-old F_1 (129SV/C57B16) recipients. Six weeks after surgery the recipients were mated and the engrafted mammary glands were harvested at day 12 of pregnancy. RNA samples in five serial dilutions were subjected to RT–PCR with primers specific for *Wnt-4* as in *A*. In parallel, RT–PCR was performed with *lacZ*-specific primers allowing normalization of the amount of transplanted epithelium. Densitometry reveals that the *Wnt-4* signal is increased threefold in the $PR^{-/-}$ *ROSA26* vs. the $PR^{+/+}$ *ROSA26* transplant. The same results were obtained in three independent experiments. (*C*) *Wnt-4* mRNA expression in cultured primary mammary epithelial cells after progesterone exposure. Primary mammary epithelial cells were cultured on collagen-coated dishes for 3 days. RNA was harvested from untreated cells and cells after 8 hr of stimulation with the progesterone agonist R5020 (20 nM) (P). Shown are two out of five serial dilutions of RNA subjected to RT–PCR with primers specific for *Wnt-4*, *Wnt-5a*, *Wnt-6*, *keratin-18*, and *GAPDH*. In each case, the undiluted RNA subjected to PCR ampliification without reverse transcription yielded no signal. Although the levels of *Wnt-5a*, *Wnt-5b*, *Wnt-6*, *keratin-18*, and *GAPDH* mRNA were unaffected by the treatment with R5020, the levels of *Wnt-4* mRNA increased two- to threefold within 4–8 hours as confirmed in eight independent experiments.

exposure. However, the expression levels of *Wnt-5a*, *Wnt-5b*, and *Wnt-6*, which are also increased during pregnancy, were unaffected by progesterone treatment. Further studies to determine whether *Wnt-4* induction by progesterone could occur in the presence of the protein synthesis inhibitor cycloheximide, were hampered by increased basal *Wnt-4* mRNA levels induced by the cycloheximide treatment, possibly reflecting cycloheximide-induced stabilization of *Wnt-4* mRNA (data not shown).

Together, our findings indicate that Wnt signaling is centrally important to progesterone-induced side-branching of the mammary ductal epithelium. In contrast, a second major morphogenetic process in the mammary gland—ductal elongation—does not appear to be mediated by Wnt signaling. In support of this, the work of others has demonstrated that the defect in ductal elongation observed in epithelia lacking the estrogen receptor is not reversed in the presence of the MMTV-driven *Wnt-1* transgene (Lubahn et al. 1993; Bocchinfuso et al. 1999).

Although we find that *Wnt-4* is the only *Wnt* gene directly induced by progesterone, it is not unique in its ability to trigger side-branching, as late in pregnancy, the ductal epithelium of $Wnt\text{-}4^{-/-}$ shows normal side-branching. We speculate that this compensation is due to the expression of other Wnt proteins later in pregnancy (Gavin and McMahon 1992; Weber-Hall et al. 1994), consistent with the notion that various Wnt proteins trigger similar biochemical responses and that their different biological functions are due to differences in their patterns of expression.

Materials and methods

Mice

ROSA26, $RAG1^{-/-}$, $Wnt\text{-}4^{+/-}$, and $PR^{+/-}$-mice were maintained on a C57Bl/6 × 129SV background. Genotyping for the β-galactosidase transgene was tested by X-gal-staining tail biopsies, PR, and MMTV $Wnt\text{-}1^{tg}$ genotyping as described (Lydon et al. 1995; Bocchinfuso et al. 1999).

Mammary glands

E14.5 embryos were harvested from crosses of $Wnt\text{-}4^{+/-}$ parents and phenotyped. The phenotyping was subsequently confirmed by PCR-based genotyping (Stark et al. 1994). The mammary anlagen were dissected and subsequently engrafted to cleared inguinal fat pads of 3-week-old recipients.

Mammary gland whole mounts, X-gal stain, and cell culture are as described previously (Brisken et al. 1998). For progesterone stimulation cells were plated on collagen-coated dishes and maintained in DMEM/F12 with prolactin (5 µg/ml) and insulin (5 µg/ml) for 3 days prior to treatment with 20 nmoles of R5020.

RT–PCR

Total RNA (1 µg) was reverse transcribed (GIBCO BRL) using random hexamers (Boehringer). Amplification was carried out by touchdown PCR using the following primers: mouse *GAPDH* (Clontech), 20 cycles; *lacZ* (Bjornson et al. 1999), 27 cycles; *keratin-18* (Schroeder and Lee 1998), 20 cycles, *Wnt-4F*, AGGAGTGCCAATACCAGTTCC; *Wnt4R*; TGTGAGAAGGCTACGCCATA, 27 cycles; *Wnt-5aF*, ACAGGCATCAAGGAATGCCAGTA; *Wnt-5aR*, AACGGGTGACCATAGTCGATGT, 25 cycles; *Wnt-5bF*, CAGAGAGTGCCAACACCAGTTT; *Wnt-5bR*, TACTCCACGTTGTCTCCACA, 22 cycles; *Wnt-6F*, CTAG-

GATGGTCGTAGACGTCCT, *Wnt-6R*, CGTTTGTGCTTTCGACAG-AG; 30 cycles.

In situ hybridization

In situ hybridization was performed as described previously (Das et al. 1994). In brief, frozen sections (14 μm) were mounted onto poly-L-lysine-coated slides and fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. The sections were prehybridized followed by hybridization with ^{35}S-labeled antisense or sense cRNA probes for Wnt4 (Stark et al. 1994) or PR (Tan et al. 1999) for 4 hr at 45°C. After hybridization and washing, the sections were incubated with RNase A (20 μg/ml) at 37°C for 20 min. RNase-A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion (Eastman Kodak, Rochester, NY). The autoradiographic exposures were from 7 to 12 days. The slides were post-stained with hematoxylin and eosin. The reddish brown grains indicate the sites of mRNA accumulation. This color is the result of lateral light scattering from the eosin staining under dark-field microscopy. Day 8 uterine sections hybridized with the *Wnt-4* or *PR* antisense probe served as positive controls, whereas sections hybridized with the sense probes served as negative controls (data not shown).

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